VOLUME II SEPTEMBER, 1897 No. 5

THE JOURNAL
OF
EXPERIMENTAL MEDICINE

# A FURTHER EXPERIMENTAL CONTRIBUTION TO THE KNOWLEDGE OF THE MECHANISM OF DEGLUTITION.

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It is now about seventeen years since Kronecker and myself* advanced the view that in the act of deglutition liquids and semi-solids are not carried down the œsophagus by the slow process of peristalsis, but are thrown down with great rapidity by the energetic contractions of the mylo-hyoid muscles especially. We supported this view, first, by experimental proofs obtained by the graphic method. A rubber balloon which was placed in the œsophagus recorded on a rotating drum two impressions during each single act of deglutition; a short one at the very beginning of the act, and a longer one which appeared the later the deeper the balloon was located within the œsophagus. There could be no doubt that the second impression was brought about by the peristaltic contractions of the œsophagus, while we had abundant reasons to interpret the first impression as coming from the rapid passing of the liquid.

But to obtain a still more striking proof of the rapidity with which the swallowed mass is usually shot down, another experiment was

* Meltzer, Ueber die Vorgänge beim Schlucken. Du Bois-Reymond's *Archiv*, 1880, 446.

made. A stomach tube was introduced into the œsophagus, the lower end with the side openings remaining a few centimetres above the cardia. Inside the tube opposite the side openings a small roll of blue litmus paper was placed with a long thread attached to it, the outer end of the thread projecting from the outside opening of the tube. When the litmus paper was rapidly withdrawn from the tube about half a second after swallowing lemonade, it was found to have turned red. This could only mean that half a second after the beginning of the act of deglutition the acid liquid was in a deep portion of the œsophagus, while the peristalsis, as we knew from our other experiments, could not reach this spot before six seconds.

Although our experiments thus far proved only that the liquid thrown down reaches the lowest segment of the œsophagus quickly, we assumed that it passes at once into the stomach. We were supported in this assumption by our observation that in the rabbit the cardia becomes momentarily relaxed at the very beginning of the deglutition, which would seem to facilitate the entrance of the swallowed mass into the stomach. Later on, however, we modified this view, when I* discovered that in the vast majority of normal individuals, about seven seconds after the beginning of a single act of swallowing, a murmur is to be heard in the region of the cardia, which has the character of a sound produced by liquid and air squeezed through a narrow aperture, while in a minority of individuals at about the beginning of the deglutition a murmur appears in the same region with a character reminding one of a sound caused by pouring or squirting liquid. I have named the former the "squeezing murmur" (*Durchpressgeräusch*) and the latter rare sound the "squirting murmur" (*Durchspritzgeräusch*). We† then took the stand that the mass thrown down is detained in the œsophagus above the cardia until the contraction of the lower section of the œsophagus begins, which occurs according to our experiments about six or seven seconds after the beginning of deglutition and with which the squeezing murmur coin-

* Meltzer, Ueber Schluckgeräusche, *Centralbl. f. d. med. Wissenschaft.*, 1883, No. 1.

† Kronecker and Meltzer, Der Schluckmechanismus u. s. w. Du Bois-Reymond's *Archiv*, 1883, Supplementband.

cides in time. We explained the origin of the sound by assuming that the contraction of the lowest section of the œsophagus squeezes the detained swallowed mass through the tonically contracted cardia, thus producing irregular vibrations which the auscultating ear perceives as a squeezing murmur. The squirting murmur occurs, we assume, in persons whose cardia for some reason or other is relaxed and in whom the swallowed mass is squirted directly into the stomach without any delay.

Our interpretation of the squeezing murmur has met with objections from several writers. Zenker* has put forward the theory that after the arrival of the swallowed mass at the cardia the air becomes separated from the liquid and remains above it, and the liquid soon oozes down noiselessly into the stomach, while the air follows later with a murmur. Why the air should follow regularly after seven seconds Zenker fails to explain, as he was not aware of the presence of the peristalsis just at this time. The theory of Zenker was accepted by Ewald† and by Quincke,‡ with the additional explanation that the air is carried into the stomach by the peristaltic wave which arrives at the lower end of the œsophagus about seven seconds after the beginning of the act of deglutition, a fact which was established by our experiments. But while Zenker's main objection to our statement was based upon the erroneous supposition that we believed the liquid to be thrown down directly into the stomach at the very beginning of the deglutition, the objection of Ewald and Quincke, on the contrary, was directed against the supposition that the liquid is carried down into the stomach by the peristaltic wave only. Both these writers have made some experiments in support of their view. I shall not enter into a detailed analysis of their statements. It suffices to say that the main object of their experiments was to show that the presence of a squeezing murmur depends upon the passing of air through the cardia and that the passage of liquid alone does not produce any sound. From this supposed fact they drew the conclusion that the

* Zenker, *Berliner klin. Wochenschr.*, 1884, p. 38.

† C. A. Ewald, Du Bois-Reymond's *Archiv*, 1886, p. 376.

‡ Quincke, *Arch. f. exp. Path. u. Pharmakol.*, xxii, p. 385, 1887.

peristalsis carries down into the stomach air only, which causes the squeezing murmur, while the liquid oozes noiselessly through the cardia before the arrival of the peristalsis at the lower end of the œsophagus. But as Quincke himself has established the fact that a murmur is present when air and liquid together are passing through the cardia, and as he insists also that air is swallowed in each act of deglutition, it is evident that their experiments are by no means conclusive for their assumption; they rather permit also the theory that the liquid remains above the cardia until the peristalsis carries liquid and air together through the aperture into the stomach, which, according to Quincke, produces a squeezing murmur. We can fully agree with Quincke that each and every act of deglutition also carries air into the stomach, and we have never insisted that the murmur is due to the passage of the liquid alone through the cardia. We have simply stated that the murmur is coincident with the contraction of the lowest segment of the œsophagus and is apparently due to the passage of the swallowed mass through the cardiac orifice, and I am willing to concede now that the presence of air in the swallowed mass is possibly the main factor in the causation of the characteristic squeezing murmur.

The interpretation of the squeezing sound and its origin are, after all, matters of only secondary importance; the main point of physiological interest is the question: Does the liquid thrown down remain above the cardia until the peristalsis carries it into the stomach, or does it ooze down previously to the arrival of the peristalsis? That it makes a halt above the cardia and is not thrown directly into the stomach just after it is squirted down the œsophagus is disputed by hardly any writer, although there is no other proof for this assumption than the presence of the squeezing murmur with its disputed interpretation.

Briefly recapitulated, the present state of the question is as follows: Our positive experiments have proven only that the fluid is squirted down into a deep part of the œsophagus. We may add here that Hamburger's deglutition murmur, which is to be heard at the back alongside the spinal column at about the beginning of the act of de-

glutition, can be followed down only to the eighth dorsal vertebra, which corresponds to a point in the œsophagus a few centimetres above the cardia. As to the further fate of the swallowed mass we have for the present no other information than the presence of a squeezing murmur at the region of the cardia just at the time of the arrival of the peristalsis. We interpreted this murmur as indicating the passage of the entire swallowed mass, air and liquid, into the stomach; the other three writers mentioned ascribe the murmur to the passage of air alone, while they assume that the liquid enters the stomach before the arrival of the peristalsis. It must be admitted, however, that neither of these views is supported by indisputable experimental proof. It is the object of this paper to offer such experimental observations as will, I believe, fully justify the stand which Kronecker and myself have taken in this question.

The observations were made on dogs and rabbits. The dogs were anæsthetized by ether and the rabbits by chloral. The main difficulty in studying deglutition in dogs lay in the strong interference of the etherization with the reflex act of deglutition. In a future communication I intend to report some observations bearing upon this point. Here, however, I shall only state briefly the method which I found to be most suitable for my purpose. After bringing the dog under thorough anæsthesia both superior laryngeal nerves were tied, tracheotomy performed, the larynx well packed with cotton and artificial respiration begun. Now the anæsthesia was reduced to that degree which permitted the return of the deglutition reflex. The state of apnœa which was brought about by the artificial respiration permits a considerable reduction of the etherization. The acts of deglutition were induced either by injecting liquid (water or milk) into the mouth or pharynx, or by faradic stimulation of the central end of one of the superior laryngeal nerves.

My first effort was directed towards getting a full view of the cardiac opening of the stomach during the act of deglutition. For this purpose the stomach of the dog was exposed, a vertical incision of about two inches made and the contents of the stomach removed. Then a wide tube with a reflecting inner surface, a vaginal speculum,

was introduced and so adjusted as to have the cardiac aperture just at the bottom of the tube. An electric light thrown into the tube facilitated the observation greatly, and the escape from the cardiac orifice of liquid or air or both could be easily scrutinized.

We knew from our former experiments that the time which passes from the beginning of the act of deglutition until the peristaltic wave reaches the cardia amounts in dogs to about four seconds. The present task was simply this: To see whether any of the swallowed liquid comes down through the cardiac orifice either abruptly at about the beginning of the act of deglutition or by slow oozing into the stomach before the peristalsis reaches there; or whether the entire swallowed mass comes down through the cardia into the stomach all at once with the peristaltic wave.

The observations were made in the first place by the graphic method, *i. e.*, one observer caused the marking of a lever upon a revolving tambour just at the beginning of the deglutition, which is easily recognizable by the characteristic sharp ascent of the larynx, while another observer, looking into the tube, marked the first appearance of liquid through the cardiac aperture. Or one observer simply called out the beginning of the deglutition and the other called out the appearance of the liquid, the length of the interval being established by a watch or simply by estimation. I have made a series of these observations on many dogs, and on each dog I made numerous single observations, and I can now state that not in one single instance was any liquid projected through the cardia at the beginning of the deglutition or did any ooze through the cardia into the stomach before the arrival of the peristaltic wave. In all cases the swallowed mass was squeezed through the cardia by the peristaltic wave, about four seconds after the beginning of the act of deglutition. When the deglutition was caused by liquid injected into the pharynx the swallowed mass consisted mainly of liquid containing only a few bubbles of air; when caused by stimulation of the laryngeal nerve the mass consisted mainly of air with only a little fluid.

A similar condition I have discovered also in rabbits. The observation is here quite simple and easy. When the stomach is lifted out

from the abdominal cavity, incised, and some of the contents removed from the region of the cardia, the cardiac aperture is plainly exposed to full view. The time for the arrival of the peristalsis at the stomach is in rabbits quite short, being a little less than two seconds. Nevertheless there could be no doubt of the fact that here, too, the swallowed mass reaches the stomach only with the peristalsis.

This line of observation led me to a search as to the exact location of the point where the swallowed mass is detained before it is carried into the stomach. We have so far said in general terms that it is detained above the cardia. That the mass does not lie immediately above the cardia we could convince ourselves by direct ocular inspection, at least in rabbits. In these animals the part of the œsophagus situated below the diaphragm is easily accessible to full view, especially when, as in the above described observations, the stomach is lifted out of the abdomen. Then we could distinctly see that the swallowed mass never entered the part of the œsophagus below the diaphragm before the arrival of the peristaltic wave.

In dogs I have often introduced the finger through the cardia into the œsophagus, even above the diaphragm, and never could I feel the presence of the swallowed mass before the arrival of the peristalsis. I have also introduced short glass tubes into the œsophagus through the cardiac orifice, thus removing the obstacles possibly caused by the tonic contraction of the cardia; nevertheless the swallowed mass did not enter the stomach before the arrival of the peristalsis.

It could be urged, however, that the finger as well as the glass tube might, as foreign bodies, cause a local contraction of the œsophagus just above them and thus create an artificial bar to the further advance of the swallowed mass. I have therefore tried a method which permits direct inspection of the lower end of the œsophagus during rest and deglutition. A cut was made in the diaphragm near its anterior insertion, through which a speculum was introduced and pushed backward in the direction of the œsophagus. After permitting the lungs to collapse for a while I usually succeeded in adjusting the tube so as to have a good view of the œsophagus just above the diaphragm. The section of the œsophagus which could be seen

in this way was a little more than an inch in length. When the artificial respiration was discontinued and the dog was still in a state of apnœa the observation was easy and distinct. In all these observations the following phenomenon could constantly be demonstrated: The œsophagus was usually in a state of medium contraction and the beginning of the deglutition caused no change in it. A few seconds later the following series of incidents could be seen rapidly to take place: First, the œsophagus was drawn upwards, then strong bulging immediately followed, and right after this a strong transversal contraction swept downward, leaving the œsophagus for a while in a contracted state. During the period of bulging air bubbles could be recognized in the water or the milk. It was clear that in none of the deglutition acts was the swallowed food directly squirted down the œsophagus to as deep a point as the region under inspection, that is, about an inch above the diaphragm.

There now arose a quite embarrassing question. The fact that liquid food is projected down into the œsophagus with rapidity was learned by observations made only on human beings. Hamburger's deglutition murmur, heard alongside the spinal column, which indicates the rapid progress of the swallowed mass, has also been demonstrated up to the present only in human beings. Although Kronecker and myself have made numerous experiments on the œsophagus of dogs and rabbits, we have made them with a view of learning other facts and not for the purpose of ascertaining whether the swallowed mass progresses downward just as rapidly in these animals as we have proven to be the case in human beings. Now we find that in dogs and rabbits the swallowed mass is not squirted down directly into the stomach. We find, furthermore, that in dogs this mass does not reach even the level of the diaphragm. Perhaps, then, in animals the food is not projected down at all, but is carried down slowly by peristalsis.

Though our old tracings obtained from the œsophagus of dogs seemed to contain some material for an answer to this question, I preferred to obtain again direct ocular demonstration of the behavior of the upper part of the œsophagus during the act of deglutition.

For this purpose I have resected in dogs parts of the three upper ribs on the left side. When the artificial respiration was discontinued and the collapsed lung pushed aside by means of retractors, the entire upper part of the thoracic œsophagus was fully exposed to view. There it could be clearly seen that, when the dog was made to swallow by pouring some liquid into his mouth and pharynx, with each deglutition the liquid shot down through the exposed œsophagus long before any peristalsis reached there. The same thing occurred when the deglutition was caused by stimulation of the nerves, *i. e.*, when only air was thrown down in the œsophagus. This observation is in so far a new fact as it had not been and could not have been observed in the human being simply because it is impossible, at least for the majority of normal human beings, voluntarily to swallow air alone without any liquid. I shall here mention a fact which otherwise does not belong in this communication, but which brought out the fact of the swallowed mass being thrown down in the exposed œsophagus of the dog with great distinctness. In the anæsthetized animals it often occurs that the first act of deglutition takes place without being followed by peristalsis. We see in such an abortive deglutition how fluid or air is shot down in the œsophagus and remains there. If a few such abortive deglutitions followed one another in shorter or longer intervals, we saw the œsophagus swell up to quite a large size, looking like a "sausage," until a peristaltic wave set in and carried the entire mass downward all at once.

The appearance of such a considerable accumulation within the œsophagus gave rise to the further question whether it was not caused simply by a mechanical obstacle, *i. e.*, whether the left bronchus was not pressing upon the œsophagus in consequence of the horizontal position of the dog, thereby preventing the swallowed mass from entering the lower part of the œsophagus and reaching the stomach. Were this assumption true, we should still have before us the task to prove that when the animal is in a normal position the food is not directly squirted down into the stomach. The following observations, however, speak positively against such an assumption. In the first place, change of position, which could be easily accomplished, espec-

ially in rabbits, did not change our results; in no position of the animal did the swallowed fluid reach the stomach before the arrival of the peristaltic wave. Secondly, the accumulation of fluid or air within the œsophagus of the dog did not cease even when the left bronchus was lifted by means of a hook from its resting place upon the œsophagus. Furthermore, I have made the following more convincing observation. A piece of the left fifth rib was resected and a speculum was inserted in a manner which permitted the observation of the œsophagus at a point half-way between the diaphragm and the bifurcation of the trachea. I have then seen how sometimes immediately after the beginning of an act of deglutition a bulging appeared at this point of the œsophagus and remained there for some time until a peristaltic wave passed by and carried it downward. The detention of the swallowed mass at this point could not be due to any mechanical obstacle. Finally, after a close observation I have sometimes found that the mass thrown down, if it was small or projected with moderate force, did not reach even the point of the bifurcation.

It deserves to be mentioned that the direct ocular observation of the thoracic œsophagus conveyed to me the impression, first, that in a state of rest the lower part seems to be tonically more contracted than the upper part; and, secondly, that the upper part is distinctly more responsive to the changes of respiration than the lower part of the œsophagus. This latter fact is in conformity with our experience with the human œsophagus. As soon as the balloon was placed in the lowest segment the respiratory oscillations became less distinct.* These observations show that the lower portion of the œsophagus is in a pronounced state of tonic contraction, which may explain the resistance offered to the mass projected downward at the onset of deglutition.

My present observations justify, I believe, the following conclusions:

In animals, as well as in human beings, liquid food is not carried down the œsophagus by the slow progress of the peristaltic wave, but is thrown down rapidly into a deep part of this canal. This applies

* Compare Figs. 17 and 18 in the table of our article, Du Bois-Reymond's *Archiv*, 1883.

likewise to the swallowing of air alone, which is brought about in animals by stimulation of the superior laryngeal nerve.

As to the depth in the œsophagus reached by the immediately projected mass, this depends upon the quantity which is swallowed, upon the force with which it is thrown down, probably also upon the degree of tonicity of the lower portion of the œsophagus, and upon other variable circumstances. In no case did the swallowed liquid immediately reach even the level of the diaphragm, to say nothing of its shooting or oozing through the cardia into the stomach before the arrival of the peristalsis. It is, therefore, an experimentally proven fact that the swallowed mass is detained for some time within the lower part of the œsophagus, and that the position taken by Kronecker and myself concerning this question is correct.

That with each act of deglutition the swallowed mass should remain for a time within the lower portion of the œsophagus must, from a teleological point of view, appear strange indeed and might perhaps be brought into causal connection with the "spontaneous" development of such pathological conditions as dilatation of the œsophagus or the formation of an œsophageal diverticulum.

I shall append here a few brief remarks bearing upon the nature of the squeezing murmur. This murmur can be heard also in the dog, as has already been stated by Quincke.* It can be heard even at a distance when the stomach is well filled with air. This is best accomplished by introducing a tube into the stomach through the duodenum. By blowing up the stomach to different degrees it can be demonstrated that the loudness of the murmur depends largely upon the quantity of air present in the stomach, *i. e.*, the larger the quantity of air, the louder is the murmur, a fact which I pointed out long ago† and which was later confirmed by Quincke. This point is to be borne in mind in examining for this murmur, as it is hardly audible when the stomach contains only very little gas.

When the tube was in the stomach and the cardiac aperture con-

* Loc. cit.

† Meltzer, Zu den Schluckgeräuschen. *Berl. klin. Wochenschr.*, 1884, No. 30.

nected only with the air in the tube, the murmur was mostly very loud and sometimes reminded one of the explosive noise from a brass instrument. The character of the murmur depends apparently not only upon the size but also upon the shape of the vibrating column of air.

When I first described the squeezing murmur I stated that it had its explanation in the irregular vibrations of the tonically contracted cardia, the air within the stomach forming the sounding board. Quincke, however, compared the murmur with the bronchial râles. Without entering into a discussion upon the adequacy of such a comparison I wish here to say that while having the cardia under direct observation, its vibrations, during the formation of the murmur, could directly be seen.

# ON THE PRESENCE OF THE AMYLOLYTIC FERMENT AND ITS ZYMOGEN IN THE SALIVARY GLANDS.

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The action of chloroform in preventing or delaying putrefaction was shown by several investigators rather more than twenty years ago. Müntz* seems to have been the first to point out that the use of chloroform water or the addition of chloroform to a liquid destroys living ferments (or a fermentation dependent upon life), but does not injure the ferments or enzymes themselves. This important bactericidal power attracted but little attention at the time, although it aroused a certain amount of opposition, which seems to have come largely from those interested in the more purely surgical aspects of the propositions advanced by Müntz. Whatever the reason may have been, but little practical use was made of the discovery for a number of years. In 1888 attention was again directed to the great value of chloroform water as a preservative liquid in which micro-organisms live with difficulty or not at all, while the ferments are not materially affected by it. The revival of interest in this useful addition to our methods is due to E. Salkowski.†

It seemed to us desirable to employ chloroform water for making extracts of the salivary glands in the hope of determining qualitatively the presence of the amylolytic ferment (ptyalin) and perhaps of its forerunner (ptyalinogen) in the gland as distinguished from the saliva.‡ We have accordingly extracted many glands of different

* Müntz, Sur les ferments chimiques et physiologiques. *Comptes rendus de l'acad. des sciences*, lxxx (1875), 1250.

† Salkowski, Ueber die antiseptische Wirkung des Chloroformwassers. *Deutsche med. Wochenschr.*, 1888. Also incidentally in his article, Ueber das eiweisslösende Ferment der Fäulnissbakterien und seine Einwirkung auf Fibrin. *Zeitschr. f. Biol.*, xxv, 92.

‡ While the existence of a zymogen in the salivary glands or in the saliva was theoretically probable, there was but little experimental evidence on

animals in this way and have tested the action of such extracts on starch paste. When an extract was found to have no amylolytic power it was treated with acetic acid for a time and the action of this acidulated extract was tested after neutralization. The substance already submitted to extraction was also treated with dilute acid and this acid extract was examined as to its action on starch. As the experiments aimed at purely qualitative results, only Trommer's test was applied, and this was found to be sufficiently sensitive for our purpose. It was of course necessary to remove the chloroform to avoid its action as a reducing substance. This was done by boiling the mixtures for some time in the water-bath. Theoretically the chloroform ought to be easily removed by this procedure. Practically, however, the result is not by any means as certain as would be expected. In many cases the chloroform seems to be held back with great tenacity by some substance in the extract, so that quite prolonged boiling is required to get rid of it completely. As there is no simple test for the presence of very small quantities of chloroform, control tubes in which the ferment had been destroyed were of great importance and were always employed in addition to the ordinary controls of plain starch paste. Even with these precautions we should be inclined to distrust some of our results if we had not made extracts with sodium fluoride (1 per cent) and found them also amylolytically active. As no suspicion can be entertained that such fluoride extracts would of themselves give a reduction with Trommer's test, as chloroform water readily does, this method proves to be of great importance.

For acidulation we have made use of acetic acid, and commonly added it so that the mixture should have an acidity of about one per

the subject. Grützner in 1876 (*vide* Pflüger's *Archiv*, xii, 306) had made statements in reference to the parotid gland of the rabbit clearly suggesting the existence of ptyalinogen in that organ. More recently, in 1886, H. Goldschmidt (Zur Frage: Ist im Parotidenspeichel ein Ferment vorgebildet oder nicht? *Zeitschr. f. physiol. Chemie*, x, 273) has shown that saliva coming from the parotid of the horse may be altogether inactive when collected with certain antiseptic precautions, and yet become active after exposure to the atmosphere. There are also experiments, notably those of Chittenden and his pupils, with reference to the influence of various substances on the amylolytic action of saliva which permit the inference that a zymogen may have been in play.

cent. This acid was chosen with the expectation that its action on the ptyalin would be less injurious than that of the mineral acids, it being well known that a very low percentage of hydrochloric acid rapidly stops the action of the ferment and destroys it. We have not examined the availability of other organic acids for this purpose. Our earlier experiments with acetic acid led us to the conclusion that extracts and gland substance may be acidulated in this way and kept at 40° C. for ten minutes without danger; while a longer exposure, even one for only twenty minutes, will probably injure or destroy the ferment. This rule was not without exceptions, but seemed definite enough to enable us to lay out a plan of work. We therefore decided upon one per cent as the limit for acidification and ten minutes as the time of exposure; the mixtures were then neutralized, added to the starch paste and placed in the water-bath. The starch paste was prepared each day by boiling one gramme of starch in one hundred cubic centimetres of water. In some few experiments a weaker acidulation ($\frac{1}{2}$ per cent) was employed, but no systematic study with reference to this point was made. On going over all our notes more carefully, after completing the research, it has become evident that acetic acid in the dilution employed by us is much less innocuous than we had supposed. We have a very considerable number of incidental observations which show very conclusively that even one per cent of acetic acid acting for only ten minutes may render an active extract absolutely inactive, and the feeble extracts seem to be particularly sensitive. Were we to take up this work again for the purpose of quantitative experimentation, as we hope to be able to do, we should certainly employ a much lower percentage of acid.

Many of the glands studied came from animals killed by chloroform in the laboratory. In a number of cases (sheep, pig, ox) the heads were brought from the slaughter-house. Where the material permitted it, the parotid glands were kept distinct from the submaxillaries, and in some large animals we were able to make a still more extensive division of the glands. As noted above, many extracts were made with chloroform water, but not a few were made also with sodium fluoride (1 per cent solution); more exactly, the proportion of observa-

tions with chloroform to those with the fluoride is about five to three. In a small number of cases (fourteen in all, ten of them being in sheep) we preserved the material in alcohol and then made our extracts at a more convenient time.

The duration of extraction varied a good deal and we were not able to follow any fixed rule. Usually the first extract was filtered off after about forty-eight hours, and a second extract made to preserve the gland substance while the first extract was tested. The extracts were prepared in this way and tested until a negative result showed the gland substance to be exhausted of its active ferment. The negative extract and also the gland substance were then examined by acidulation for the presence of the forerunner of the ferment, ptyalinogen. The method was altogether qualitative and has all the unsatisfactoriness and incompleteness of such an analysis. We were fully cognizant of the objections which may be urged, but we were unable at the time to carry out the investigation quantitatively.

The results may be summarily presented in a brief account of them for each group of animals studied.

I. *Dog.* All the glands of one of the animals were extracted together with chloroform water. The different glands of the remaining eight sets were kept apart, that is, the parotids were separated from the submaxillaries. In three of the eight chloroform extracts were made, while in another series of three, one of each pair of glands was put into chloroform water and the other into the fluoride solution; the remaining two sets of glands were extracted with distilled water. In this way it was shown that while there may be slight differences in a pair of glands, they are usually alike as to containing an amylolytic ferment or none. The division of the glands also permitted a comparison of the action of the chloroform with that of the fluoride and showed that the method employed to remove the former was trustworthy.

In two of the dogs examined no active ferment was found in extracts of the parotids or the submaxillaries. These extracts contained no zymogen, but the substance of one pair of parotids and also of the corresponding submaxillaries gave a very active extract after treatment with acid.

Six other parotids and five submaxillaries contained the ferment, and an active extract was also obtained from the mixed glands already mentioned. So far as a purely qualitative analysis permits us to judge, the submaxillary of the dog usually yielded a decidedly weaker extract. In one set of parotid glands (in chloroform and also in fluoride) acidification produced an active ferment in the extracts which were previously negative, because the ptyalin had been removed in the first extraction, but had no action on the extracts of the submaxillaries of the same dog. The exhausted gland substance (parotid) in this experiment also contained a small amount of the zymogen.

The water extracts call for a word of comment. One set has already been mentioned among the negative first extracts; the remaining parotid contained an active ferment, while the results with the submaxillary were altogether negative. All these water extracts, as well as a second set of water extracts of the glands, became malodorous on account of putrefaction, and, in consequence of this, one negative extract of a submaxillary became active. As the other extracts remained inactive, we may assume that the extract in question became active because putrefaction transformed an inactive zymogen, and not because amylolytic micro-organisms developed in it. In the fluoride extracts of one of the dogs a similar change was noticed, the extracts of both the parotid and submaxillary glands becoming potent after standing for some days, although not distinctly ill-smelling. It is therefore apparently just to conclude that putrefaction sometimes, although not always, transforms an inactive material in a salivary gland into an active form.

*Cat.* Extracts were made with the salivary glands of eight animals. In two cases no separation of the glands occurred. From one set of these an active extract was obtained by using chloroform water. From the other set, which had been atropinized, negative results were obtained both in the original extracts and after treatment with dilute acid.

The glands of the other six cats were kept separated. Of the six pairs of parotids, four gave up a small amount of ferment to fluoride or chloroform, and in two pairs further treatment of the extracts with

acetic acid increased the activity or, in one of them, rendered an inactive extract active. It is also of interest to note that the best results were obtained with fluoride extracts. The infusions of the submaxillary glands contained a minimal amount of the amylolytic ferment in two instances, but distinctly less than was found in the corresponding parotids. In one submaxillary extract a small amount of ptyalinogen was demonstrable. The acidification of the gland substance resulted negatively in four animals, but was effective in two.

Several kittens, two to three months old, were also examined. In one set of mixed glands, atropinized, a trace of ferment was found; and this was not increased by treatment with acid. The other set of glands was altogether negative. Another pair of kittens also gave totally negative results for extractions with fluoride after the glands had been kept in alcohol (95 per cent) for about seven months. This may have been due to the action of the alcohol, for we have a certain amount of evidence that this form of preservation tends to destroy the amylolytic ferment or in some peculiar way to render it inert. We had an experience of this kind with one of the cats whose glands gave active extracts with fluoride, but quite negative ones where the corresponding glands had been kept in alcohol for thirty days.

*Pig.* The glands of two animals were placed in sodium fluoride, chloroform, or alcohol. The parotids were all exceedingly rich in ptyalin, but the amount seemed to vary somewhat in the two animals and even in the two glands of the same pig. One of the active fluoride extracts came from a gland which had been in strong alcohol for ten days. In this case, at least, alcohol had not destroyed the ferment, although it may have weakened it. The submaxillaries gave feeble infusions, the amylolytic action being in each case very much weaker than that of the corresponding parotids. Treatment of the later inactive extracts, as well as of the gland substance, with dilute acid in the usual way led only to negative results.

*Opossum.* We had an opportunity to extract the glands of four animals. One was killed in December, the remaining three in May, at a time when hibernation seemed to be fully ended. In no case was a really active ferment obtained by extraction with sodium

fluoride or chloroform water; in fact only the December observations gave us any positive results at all, and here the action was exceedingly feeble, being distinct in the case of one parotid extract after eighteen hours. No evidence of a zymogen was obtained.

*Rats and mice.* A limited number of experiments were made with the glands of two rats and of several mice, those of the latter being treated together as from one animal. Both the parotids and submaxillaries gave extracts of very notable amylolytic power and proved to be unusually tenacious of the ferment, so that successive extractions lasting even five, twenty-one and twenty-four days respectively did not completely exhaust the glands. No evidence of a zymogen was obtained.

*Rabbits.* The glands from thirty-two animals were examined in various ways. In many instances death was caused by a quick blow on the occiput, in order to avoid any stimulation of the salivary secretion arising from the use of an anæsthetic. The extracts were made in part with chloroform water and in part with sodium fluoride. In six cases the glands were first placed in strong alcohol (95 per cent) and then treated with sodium fluoride or, in one instance, with chloroform water.

The extracts made from fresh parotids or from mixed glands all contained the amylolytic ferment, except in one group of very young rabbits, five days old, whose glands gave quite inactive extracts. The infusions of the submaxillary glands alone were much less potent, nearly one-third of them yielding no ptyalin. No distinct difference was noticed between chloroform and fluoride as an extracting medium. In eleven cases atropinization did not seem to affect the results.

The submaxillary of the rabbit is also much more easily exhausted, and generally a second extraction for one day was sufficient after a first extraction of one or two days. The parotid is decidedly more tenacious of the ferment or richer therein; even after two extractions for twenty-four to forty-eight hours a third extract was found to be active.

The glands which had been in alcohol for five to eight months contained much less active ferment, as shown by the amylolytic power of

the extracts, and this effect of the alcohol was much more pronounced in the submaxillaries. On the other hand the action of alcohol for five days did not materially affect the extracts.

The existence of ptyalinogen was tested in many of the extracts which had been found to be inactive and also in the substance of the glands from which such extracts had been made. Treatment with acetic acid increased the activity of the extract of one parotid gland and we have six positive results with otherwise inactive extracts of submaxillaries. The examination of the substance of the exhausted glands proved to be much more interesting, and ptyalinogen was found in sixteen cases, of which nine were submaxillaries and four parotids, while three were mixed glands.

*Ox.* Here our experience is somewhat limited, being confined to an examination of the glands of one ox and of a calf. The extracts were made with chloroform water and also with sodium fluoride. One submaxillary of the ox contained a small amount of an active ferment, while in the other only a trace was found. None of the glands from the ox gave evidence of the presence of ptyalinogen after extraction. One plain extract, however, became decidedly more active after being kept at 40° C. for five hours. The glands of the calf yielded no active ferment, but evidence of the zymogen was found in one parotid after treating the substance with acetic acid in the usual manner.

*Sheep.* Our observations were made on a considerable number of sheep, the glands of seventeen being used for various purposes. Seven sets of glands were placed at once in alcohol and thus no simple first extracts were made from them. In the ten sets of plain extracts which were studied, an active amylolytic ferment was found for only one sheep, and then the ptyalin was present in exceedingly small amounts, the reaction being noted as merely a " trace " for the fluoride extracts. After treating such material with acid an active ferment was found to have been formed in the extracts of two submaxillary glands. The substance of the glands of the same group, after infusion with acetic acid, gave positive results for two parotids and for two submaxillaries, one of each coming from the same sheep.

The alcohol material was of necessity preserved somewhat irregu-

larly; two sets of glands were in alcohol for five months, the others for periods varying from four to twenty-eight days. The extracts were prepared partly with chloroform and partly with sodium fluoride; the single glands of three pairs were extracted with both liquids. None of these extracts after alcohol showed any trace of the active ferment. A small amount of the zymogen was found, however, in the extracts of two pairs of glands (parotid and submaxillary) which had been in alcohol only a little over twenty days. In the substance of two submaxillaries, after exposure to alcohol for four and twenty days respectively, the ptyalinogen was also clearly demonstrable. We have, then, altogether eight different sheep in some of whose salivary glands the zymogen was found.

When this investigation was first undertaken we had what seemed to us the good fortune to meet with a number of successful experiments. This was so inspiring that we probably failed at the time to appreciate the full significance of such negative results as were met with. We did not really get out of this mental rut until the research had reached a point where we were obliged to leave it for a time on account of other occupations. Despite the danger of the method employed, since the brief use of even one per cent acetic acid may injure ptyalin and ptyalinogen, and since this danger is probably greater when the amount of the ferment is small, the results seem to us to demonstrate beyond question that a zymogen exists in many salivary glands. Our observations show this convincingly for the dog, cat and sheep, and less clearly for the ox. The negative results for the pig, opossum and rat may be due in part to insufficient material and in part to the method which we have employed.

# ON THE PRESENCE OF A MILK-CURDLING FERMENT (PEXIN) IN THE GASTRIC MUCOUS MEMBRANE OF VERTEBRATES.

A SUMMARY OF EXPERIMENTS MADE IN THE BIOLOGICAL LABORATORY OF BRYN MAWR COLLEGE BY CAROLINE W. LATIMER, M. D., ANNE C. COLEMAN, B. A., AND ROSALIE A. FURMAN, B. A.*

BY JOSEPH W. WARREN, M. D.

The experiments noted in this paper were undertaken to test the value of chloroform water for making extracts of the gastric mucosa. As material accumulated we became interested in the examination of the stomachs of various accessible vertebrates for the milk-curdling ferment. The results show very convincingly that this ferment may be considered to exist in all the subdivisions of this great group of animals. This conclusion is not presented as an entirely novel proposition, but rather as a somewhat detailed confirmation and extension of the work of other investigators. More than twenty years ago the wide distribution of the ferment in question was established, in general terms, by Hammarsten in a notable article in which he showed conclusively that milk is curdled by a special *ferment* in rennet, and not merely by the action of an acid.† Hammarsten says that he found the ferment, or the proferment, in the gastric mucous membrane of every animal examined. He does not explicitly specify what animals were studied, but the inference may be made that he refers only to vertebrates, since he mentions "mammals, birds and fishes."‡

* Some supplementary and confirmatory observations were also made by Misses A. F. Blauvelt, H. W. Cadbury, and C. F. McLean.

† Olof Hammarsten. Om mjölk-ystningen och de dervid verksamma fermenterna i magslemhinnan. *Upsala Läkareförenings Förhandlingar*, viii, 1872, p. 63; and also his own very full report of this article (Ueber die Milchgerinnung und die dabei wirkenden Fermente der Magenschleimhaut) in Maly's *Jahresber. üb. d. Fortschr. d. Thierchemie*, ii (Lit. 1872), 118.

‡ Reprint of the Swedish article, p. 16.

It is perhaps worthy of notice in this connection that a knowledge of the possession of the curdling power by many animals besides the mammals is not as new as is commonly supposed. Thus Spallanzani, more than a cen-

He was able to show that the curdling ferment could be extracted from every gastric mucosa by means of acidulated water, while an

tury ago, notes that "this is well known to cooks, who, when they have no rennet, take the innermost coat of the stomach of a fowl and [after washing it] steep it in water; which water, when thus impregnated with the juice of the stomach, will serve for turning milk as well as rennet itself." Spallanzani says that he made such water extracts of the stomachs of a great variety of gallinaceous birds and demonstrated their curdling power. He further "discovered that this property belongs also to intermediate and membranous stomachs, by experiments on that of the crow, heron, birds of prey, the rabbit, the dog, cat, various reptiles, and several scaly fishes." He also found that dried stomachs, "chiefly taken from the gallinaceous class," retained this power for at least three years. Spallanzani believed that the curdling was not due to an acid, and his method of experimentation would seem to exclude the free acid of the gastric juice. [Spallanzani. Dissertations relative to the Natural History of Animals and Vegetables. New edition, London, 1789, vol. i, 332-337. Also vol. ii, 702 of Senebier's edition: Opuscules de physique animale et végétale (etc.), Pavie, 1787.]

Home, too, observed that milk may be coagulated by rennet made, not only from the membrane of the hog's stomach, but also from the crop and gizzard of a cock, or the stomach of the shark, salmon, and thornback. By rennet he seems to mean an infusion of the dried membrane. He also had positive results with fresh material, using the cardiac portion of a chicken's stomach and the horny lining of the gizzard or the cardiac portion of the stomach of the hawk and the turkey. [Sir E. Home. Experiments to ascertain the coagulating power of the secretions of the gastric glands. *Phil. Trans. Roy. Soc. Lond.*, 1813, p. 96.]

John Hunter, too, in his "Observations on Digestion" [vol. iv, p. 104, of Palmer's edition of his Works, London, 1837], says: "I have found this coagulating power in the stomach of every animal that I have examined for that purpose; from the most perfect down to reptiles [meaning, according to Owen's note, probably 'creeping things,' as insects and worms]; and in the appendages which I have considered as only reservoirs preparatory to digestion (as the first stomach in the ruminating animal, and the crop in birds), I have discovered no such power." It is, however, not improbable that Hunter refers only to the action of such liquid as he found in these stomachs even when digestion was not going on.

Tiedemann and Gmelin [Die Verdauung nach Versuchen, 1826] mention the experiments of the writers quoted above as well as those of Verrati and of Réaumur (for birds, ii, 131-137), and of Andry (for the pike, ii, 269), but see in them only evidence that the digestive juices contain acid. This idea that the coagulation of milk depends upon the action of an acid continued to prevail down to the time when Hammarsten showed conclusively that a ferment is in play. There was, however, plenty of other evidence that a "free" acid is not esential to the clotting process. This had been shown by Mitscherlich [see Mulder: Versuch e. allg. physiol. Chem., ii, 1015], and particularly by Selmi in a preliminary communication [Recherches sur l'action de la présure dans la coagulation du lait. *Journ. de pharm. et de chimie* (3), ix, 1846, 265], as well as by Deschamps in 1840.

extract made with unacidulated distilled water contains a substance which does not curdle milk, but which will form the curdling ferment when acted upon by very dilute hydrochloric acid. As a name for this new ferment "Löpe" was proposed by Hammarsten in his Swedish article and "Lab" in his German abstract. "Löpe" and "Lab" are, however, as Hammarsten well knew, merely the old names in these languages for milk-curdling extracts made from stomachs, and are the equivalents of the French "présure" and the English "rennet," which also mean the extract and not the ferment itself. Since the recognition of a curdling ferment became general various names for it have been in use. In German it is called the Labferment, and this term has even been used freely by the most conspicuous French writer on milk coagulation. English usage has presented no uniformity until quite recently. A few years ago the best and most facile writer on physiological topics in English introduced the word "rennin" into his admirable text-book, and this word has met with considerable acceptance. The only thing that can be said in favor of the word is that the form seems to permit the construction of renninogen to describe the mother substance or forerunner of the ferment. Labferment does not admit properly of such a termination, and so the form proferment or some circumlocution has to be adopted. No one has been bold enough as yet to say "Fermentogen" or "Labfermentogen," despite the catholicity of much of modern scientific and especially medical nomenclature.

It is perfectly possible to find a suitable word for this ferment analogous in its origin to the names used in describing other ferments and having, in a sense, the sanction of our most ancient professional writers. Such a word is *pexin*, formed from the Greek πῆξις (as pepsin is formed from πέψις), and used by Pagès about nine years ago.* There is also another word, chymosin, which has a suitable form. This was proposed many years ago by Deschamps, who recognized a peculiar substance in the gastric juice as the material on which its action depends. This writer dwells upon various peculiarities of chymosin, and his services in establishing the essential conditions of

* Pagès. Recherches sur la pexine. Thèse de Paris, 1888.

its power apparently have not received the recognition which they deserve.* He showed, for example, that the curdling of milk by what we should now call the ferment may occur in an alkaline medium. Chymosin did not meet with favor as a name, probably because it was held by many to be really identical with pepsin, which had been discovered a short time before.

There can be little question that it is desirable to have a good word of suitable form as a name for the ferment whose existence is admitted despite differences of views as to the manner in which it acts. Pexin meets all the requirements of the case, resembling in structure many other words used as names of digestive ferments (ptyalin, pepsin, trypsin, steapsin or pialyn), and forming a convenient name for the forerunner or proferment (pexinogen, like pepsinogen and trypsinogen) whose existence is also generally accepted. Pexin has an historical advantage because of its relation to words used by ancient writers to designate coagulation. It will suffice for the purposes of this article to point out that such words were used by Galen in the second century, and also by Aristotle and Hippocrates perhaps five hundred years earlier.† We shall make use of pexin and pexinogen

* Deschamps. De la présure. *Journ. de pharm. et des sc. access.*, 1840, xxvi, 412.

He also found the peculiar material in the mucous membrane of the glandular stomach of gallinaceous birds, but not in the crop or gizzard. He infers that it belongs to the stomach of all animals, but admits this to be a free generalization ("ne . . . que par induction"). Besides observing that milk is clotted by rennet even after the addition of bicarbonate of soda so as to make it alkaline, Deschamps has also experiments which show clearly that a feeble rennet was improved by the action of sodium chloride. He really had the evidence of the existence of a proferment, but did not know how to interpret his results. There is a fairly good abstract of the article in Schmidt's *Jahrbücher*, xxix (1841), 170.

† The ancients, as I learn from those who are more familiar with their works, knew that milk could be coagulated by certain plant juices and by the curdled milk in the stomachs of various sucking animals. This latter material is the πυετία or πῆγμα which the dictionaries sometimes translate as "Lab" or "rennet," although rennet, in the modern sense, was probably unknown. The verb πήγνυμι is frequently used to describe the curdling of milk and the clotting of blood. Other verbs signifying solidification, or making firm, or stiffening (as does πήγνυμι) are also used for this purpose. The caseification of milk is sometimes more particularly designated by τυρεύω.

From Galen the following citations, according to the volumes and page

in this article to designate the active form of the ferment that clots milk and the inactive form, or forerunner, respectively. Should it seem desirable to use pexin for a curdling ferment in general we might form hæmopexin and galactopexin* to designate the different kinds, bearing in mind, however, that we have as yet no good reason to consider these ferments to be in any way related.

It is proper to add here that Grützner† has found the curdling ferment in the glands of the œsophagus of the frog, but not in the

of the edition by Kühn in 1826, may be noted. In the treatise "De simplicium medicamentorum temperamentis ac facultatibus" he has a chapter (x, 9) about cheese which he says he adds to his remarks on milk because cheese is coagulated milk (*γάλα πεπηγός ἐστιν*) [vol. xii, p. 269]. In the same chapter he speaks of the cheese as separated from the serum *κατὰ τὴν πῆξιν* [p. 270]. Other references are: *πηγνυμένῳ γάλακτι* [iv, 632]; *παγῆναί τε αὐτὸ καὶ τυρωθῆναι κατὰ τὴν γαστέρα* [vi, 684]; *ἢ διὰ πυτίας πηγνύμενον* [vi, 694]; *τυροῦται* and *τυροῦσθαι* [x, 475].

For Aristotle many quotations might be made from sections 5-7 of Bk. III, of the Meteorologica [ed. Bekker, 1831, vol. i, 382-4] where *πῆξις* and *πήγνυμι* are used for various forms of solidification, incidentally also for that of blood and milk. The following citations will confirm the usage: *De animalium historia*, iii, 5 [Bekker, i, 515]: *τὸ αἷμα . . . πήγνυται.* In the next chapter [p. 516] he compares the coagulation of the blood of some animals with that of milk:—*ἐστι δ'ἀμφοτέρων αὐτῶν ἡ πῆξις οἱ στιφρά, . . . καθάπερ ἡ τοῦ γάλακτος, ἂν τις εἰς αὐτὸ τὸ πῆγμα μὴ ἐμβάλλῃ.* In chap. 20 he has much to say about milk and its coagulation (*γάλα πήγνυται*) and caseification (*τυρεύεται τὸ γάλα*), and he mentions explicitly the action of fig juice and "rennet":—*πήγνυσι δὲ τὸ γάλα ὀπός τε συκῆς καὶ πυετία* [i, 521-2]. See also ii, 4 and iii, 15 in the treatise *De partibus animalium* (p. 66 and p. 176 of the edition of von Frantzius, Leipzig, 1853). Two passages in his treatise *De generatione animalium* are particularly valuable. One is in chap. 20 of Bk. I. [Bekker i, 729]: *οἷον ἐν τῇ τοῦ γάλακτος πήξει τὸ μὲν σῶμα τὸ γάλα ἐστίν, ὁ δὲ ὀπός, ἡ πυτία . . .* In the second [iii, 11; B. I. 762] *πῆξις* has another sense: *. . . καὶ πεπηγυῖαν τὴν αὐτὴν πῆξιν τοῖς ὀστοῖς καὶ τοῖς κέρασι.*

The "works of Hippocrates" do not give much information about the clotting of milk if we may trust the indices of the editions of Foësius and of Littré. *Πήγνυμι* and even *συμπήγνυμι* are used for the coagulation of blood (Littré vi, 189, 204; viii, 594). The dictionaries also contain *σίμπηξις*, and note Homer as using the verb in connection with milk [*γάλα συνέπηξε*, Il. 5, 902].

* Several years ago the opinion of Prof. Goodwin was asked as to the form of these words. He kindly pointed out that, while hæmopexin is perfectly justifiable, galactopexin is rather an awkward compound, though not worse than many modern ones. Prof. Goodwin also suggested that a good word might be made from the poetic word for milk, *γλάγος*, found in Homer, out of which the (poetic) adjective *γλαγοπήξ* was actually formed.

† Pflüger's *Arch.*, xvi, 1877-8, 119.

mucosa of the stomach. Langley,* on the other hand, found the zymogen in both places in the frog, besides demonstrating its existence in the stomach of the dog, cat, mole and rabbit.

In preparing our extracts the mucous membrane was removed from the muscular coat and carefully washed. It was then cut into small pieces, rubbed up with sand in a mortar, and placed in a considerable volume of saturated chloroform water for extraction. No exact rule was adopted as to the proportion of liquid to the quantity of mucous membrane, and in a few instances the extracts were possibly too dilute. When the stomachs were very small or the muscular layer was very thin no effort was made to separate the mucosa, but the entire stomach was cut up. The material came in many cases from animals killed in the laboratory; in others it was brought from the slaughter-house. For the opportunity to procure the stomachs of sea fishes we are indebted to the courtesy of Messrs. Oscar and Cedric Laighton, of the Isles of Shoals, off the coast near Portsmouth, N. H.

It is evident that under these varying conditions the membranes were often put into the chloroform water several hours after death. The general uniformity of our results shows, however, that such delay involves no considerable risk of a transformation of the pexinogen on account of putrefaction. In one case (No. 55 of the table) this question was tested by leaving half a mucous membrane exposed to the air in a warm room for five hours.

The extract obtained in this way, and which for the sake of brevity may be called the "chloroform extract," is usually a somewhat opalescent liquid of a slightly yellow or very faintly red color. There is often a considerable admixture of mucus despite careful washing; this is especially noticeable in extracts of the stomach of the pig, and such extracts do not filter readily.

Such chloroform extracts keep pretty well. By this is meant that there was usually no distinct evidence of putrefaction either in the appearance or in the odor of the liquid. These are of course very insufficient tests of the complete absence of putrefaction, but they are

* *Jr. Physiol.*, iii, 269.

sufficient for our purpose. The observations of Grünert show that similar extracts may be actually sterile in the bacteriological sense.* The protection afforded by chloroform is far from being unlimited, as has been abundantly shown by others. Some of our extracts became bad in a few weeks, while others remained good for many months. This probably depends upon the proportion of chloroform, that is to say, upon the concentration of the extract, but we have no exact information to offer on this point. In certain cases, particularly where the amount of chloroform water seemed rather small, a few drops of chloroform were added to ensure saturation. It has been shown that chloroform hinders the artificial digestion of fibrin by pepsin and the coagulation of milk by commercial rennet. The delay thus caused is, however, not very pronounced and requires larger amounts of chloroform than were used in our experiments.† Small amounts of chloroform are said to act favorably and to quicken the work of the ferments.

The transformation of the inactive pexinogen into the active pexin was brought about by acidulation with acetic acid in such an amount that the resulting mixture contained one per cent of the acid, no allowance being made for possible loss of acid by combination with substances in the extract. Hammarsten in his original work on the ferment of coagulation acidified his extracts with hydrochloric acid so added as to cause an acidity of one-tenth per cent. In view of the fact that the mineral acids had been thought to act destructively on some ferments, and also because our acidulated extracts often had to be prepared many hours before they could be tested, we decided to use acetic acid as less likely to injure the ferment in question. Our experience shows that acetic acid readily transforms the pexinogen, and also that such acidulated solutions may be kept for a considerable length of time without any marked injury to the curdling power of the ferment. It may be added that we have occasionally tried the

* A. Grünert. Die fermentative Wirkung d. Dünndarmsaftes. Inaug. Diss., Dorpat, 1890, 23.

† Dubs. Der Einfluss des Chloroforms auf die künstliche Pepsinverdauung. Virch. *Arch.*, cxxxiv (1893). 519.—Benjamin. Labgerinnung. Ibid., cxlv (1896), 30.

action of hydrochloric acid in these experiments and have grown sceptical in regard to the common opinion of its deleterious action. We hope to examine this point more fully at another time.

The acidulated extracts were allowed to stand for a number of hours at the temperature of the room. We have not looked carefully into the question of the time and temperature best suited to the transformation, but have found that if the mixture stand over night (12–24 hours) the desired result is readily attained. Such observations as we have show that this action may be hastened by a higher temperature, say 40° C., and that a more dilute acid may be efficient.

The extract was then neutralized by the addition of a convenient alkali. In most of the experiments with which this paper is concerned we have added sodium carbonate in substance in order to avoid further dilution of the extract. This method has its inconveniences, because it is not easy to determine the point of neutralization exactly by means of the ordinary test papers. It has, to be sure, been noted by Hammarsten and others that clotting occurs readily even in faintly alkaline mixtures, but our experience has been that even a slight degree of alkalinity very much lessens the activity of the ferment in such solutions as we employed and may lead to absolutely negative results. It is probable that Hammarsten used more concentrated extracts. This view seems to be confirmed by the fact that we also had good clottings with an alkaline reaction in our earliest experiments, where our extracts were presumably more concentrated than they were later when a growing greed of experimentation demanded a larger supply of material. There is reason to think that the injurious action of alkalies stands in some peculiar relation to the concentration of the solution of the ferment. To this question we hope to return at another time. It should be added that the favorable influence of a faintly acid reaction has been noted by various observers, particularly by Arthus.* This neutralized or very faintly acid extract was then

* This refers to a degree of acidity so slight that there is no danger of an acid coagulation or precipitation, as has been shown by numerous control experiments. See the articles by Arthus and Pagès in the *Arch. de physiologie*, xxii (1890): Recherches sur l'action du lab et la coagulation du lait dans l'estomac et ailleurs, p. 331; Sur le labferment de la digestion du lait, p. 540;

added to milk, usually in the proportion of one part of extract to two of milk, and the tubes were placed in the digestion apparatus and kept at about 40° C. Suitable controls of plain milk and of mixtures made with boiled extracts were also provided. The observation lasted ordinarily from thirty minutes to an hour and a half; those coagulations which occurred after this lapse of time were considered less satisfactory, and such experiments were usually repeated or modified to confirm the results. A very small number of extracts were prepared by using a solution of sodium fluoride (1 per cent) instead of chloroform water, but no extended test of this substance was undertaken.

The results of our experiments, so far as they relate to the general question of the distribution of pexinogen among the vertebrates, are put in tabular form below. Each animal in the table bears a number which refers to the memoranda about it and indicates nothing in reference to the time relatively to other experiments. In the second and third columns are noted the results of the action of the chloroform extracts (" plain extracts ") on milk and also on fibrin in dilute hydrochloric acid, the mixture having an acidity of 0.2 per cent HCl. The fourth column gives the action on milk of the extracts which have been acidulated with acetic acid for a suitable period and then neutralized (" Extract + $\bar{A}$ "). The signs + and 0 are used to indicate that the results were positive (curdling of the milk, or solution of the fibrin) or negative, while a simple line (—) merely means that the corresponding experiment was not tried with that particular extract. It should be added that each record only gives the general result; it may, and indeed usually does, represent many observations made in very numerous experiments. In a column of " remarks " various comments are added whose significance is obvious or will be discussed further on.

and also one by Arthus: Le labferment est un élément constant de la sécrétion gastrique des mammifères adultes. *Comptes rendus de la soc. de biol. de Paris*, 1894, 178. Ringer has also mentioned the importance of feeble acidification in testing coagulability.

TABLE I.

SHOWING THE ACTION OF THE PLAIN CHLOROFORM-WATER EXTRACT ON MILK AND FIBRIN, AND THAT OF THE SAME EXTRACT ON MILK AFTER TREATMENT WITH AN ACID AND NEUTRALIZATION.

| No. | Plain Extract. | | Extr. + Ā. | Remarks. |
|---|---|---|---|---|
| | Milk. | Fibrin. | Milk. | |
| I. | | | | I. Skate. Barn-door skate (Raja laevis). |
| 1 | 0 | + | + | |
| 2 | 0 | + | + | |
| 3 | 0 | — | + | |
| 82 | — | + ? | 0 | 82 gave uncertain results when neutralized with $CaCO_3$. |
| II. | | | | II. Mackerel shark (Lamna cornubica ?). |
| 6 | 0 | + | + | |
| III. | | | | III. Dog-fish (Squalus acanthias). |
| 79 | — | — | 0 | 79 and 80 also 0 for various combinations with $CaCO_3$ and $CaCl_2$. |
| 80 | — | — | 0 | |
| 81 | 0 | — | 0 ? | |
| IV. | | | | IV. Cod-fish (Gadus morrhua). |
| 7 | 0 | + | + | |
| 8 | 0 | 0 | + | |
| 85 | 0 | 0 | + | |
| 86 | 0 | 0 | 0 ? | 86 was + ? for $CaCO_3$. |
| V. | | | | V. Hake (Merluccius bilinearis). |
| 10 | 0 | — | + | |
| VI. | | | | VI. Pollack (Pollachius virens). |
| 11 | 0 | + | + | |
| VII. | | | | VII. "Monk-fish" ("Angler": Lophius piscatorius). |
| 83 | 0 | — | 0 | 83. Acidulated extr. was + with aid of $CaCl_2$. |
| 84 | 0 | 0 | 0 | |
| VIII. | | | | VIII. "Tailor" (Pomatomus saltatrix). |
| 12 | 0 | 0 ? | + ? | Extr. gave uncertain results, but treatment of *substance* with acid and $CaCO_3$ gave an active extract. |
| IX. | | | | IX. Frog. All "winter" frogs from tank. |
| 17 | 0 | + | + | |
| 19 | 0 | + | + | 19. Stomachs of 14 frogs in chloroform water. |
| 87 | 0 | — | [+] | 87. 5 stomachs. Gave + result with $CaCO_3$. |
| 16 | 0 | — | — | Scraps of mucous membrane added to milk. No extracts made. |
| 18 | 0 | — | — | |
| X. | | | | X. Turtle. (Pseudemys rugosa ?). |
| 20 | 0 | — | + | |
| 21 | 0 | — | + | |
| 22 | 0 | + | + | |
| 23 | 0 | — | + | |
| 24 | 0 | — | 0 | 24. An old extract kept 5½ months. |
| 24*a* | 0 | — | + | 24*a*. The plain extract became + in 3½ weeks, but even then was more active after treatment with acid. |

TABLE I.—*Continued.*

| No. | Plain Extract. | | Extr. + Ā. | Remarks. |
|---|---|---|---|---|
| | Milk. | Fibrin. | Milk. | |
| 25 | 0 | 0 | [+] | 25. + for $CaCO_3$. |
| 26 | 0 | + | + | |
| 27 | 0 | 0 | 0 | 27. Also 0 for $CaCO_3$. |
| XI. | | | | XI. Chicken. |
| 30 | 0 | ? | + | Two or three. In 30 & 31 no separation of the stomachs was made. |
| XII. | | | | XII. "Partridge" (Ortyx virginianus?). |
| 31 | 0 | + | + | Three stomachs. |
| XIII. | | | | XIII. Pigeon. |
| 29 | 0 | ? | + | Proventriculus. The crop was also + (for $CaCO_3$). The extract of the gizzard was altogether negative. |
| XIV. | | | | XIV. Snake. |
| 32 | 0 | — | [+] | + for $CaCO_3$. |
| XV. | | | | XV. Opossum (Didelphys virginiana). |
| 33 | 0 | + | + | |
| 36 | 0 | + | + | |
| 34 | 0 | + | + | Fluoride extract. |
| 35 | 0 | + | 0 | " " |
| XVI. | | | | XVI. Rat. |
| 37 | 0 | ? | 0 ? | 37 was + for $CaCO_3$. Stomach full. |
| 38 | 0 | + | + | 38. HCl used here instead of acetic acid. |
| 88 | — | + | — | |
| 89 | — | + | 0 | 89. HCl. 0 for $CaCO_3$. |
| 90*a* | 0 | — | [+] | Stomach partly full. + for $CaCO_3$. |
| 90*b* | — | — | 0 ? | Stomach full. ? for $CaCO_3$. |
| 90*c* | 0 | — | [+] | " " + for $CaCO_3$. |
| 90*d* | 0 | ? | 0 | " " |
| 90*e* | 0 | + | [+] | " " + for $CaCO_3$. |
| 90*f* | 0 | ? | [+] | " " + for $CaCO_3$. |
| 90*g* | 0 | + | 0 | " " |
| 90*x* | 0 | + | + | Stomach empty. |
| XVII. | | | | XVII. Mouse. |
| 39 | 0 | + | 0 ? | Number not noted. ? in 75 minutes. |
| 40 | 0 | + | 0 | " " " Acidulated for 5 days. |
| 41 | 0 | traces | 0 | " " " Very young and full of milk. Acidulated for 5 days. |
| 42 | 0 | 0 | 0 | Two stomachs' full. ? for calcium compounds. |
| 43 | 0 | 0 | 0 | |
| XVIII. | | | | XVIII. Rabbit. |
| 44 | 0 | + | + | Acidulated 3 days, and also 10′ at 40° C. |
| 45 | 0 | + | + | Acidulated 3 days. |
| 46 | 0 | 0 | + | Stomach full. |
| 47 | 0 | 0 | [+] | 2 young rabbits. + for $CaCO_3$, but 0 about three weeks later. |
| XIX. | | | | XIX. Sheep. Fourth stomach. |
| 48[1] | 0 | + | + | Fundus portion. |
| 48[2] | 0 | + | + | Pylorus " |
| 49[1] | 0 | — | + | Fundus. Plain extract became + in 5 days without evident putrefaction. |

TABLE I.—*Continued.*

| No. | Plain Extract. | | Extr. + Ā. | Remarks. |
|---|---|---|---|---|
| | Milk. | Fibrin. | Milk. | |
| $49^2$ | 0 | + | 0 | Pylorus. Plain extract was 0 after 3 weeks. |
| $50^1$ | 0 | — | + | Fundus. Plain extract became + like $49^1$. |
| $50^2$ | 0 | — | + | Pylorus. Plain extract + in 5 days and bad. |
| XX. | | | | XX. Ox. Fourth stomach. |
| 51 | 0 | + | + | Plain extract 0 after 14 days. It was + when tested a year later, but even then treatment with acid quickened its action very much. |
| $53^1$ | + | + | — | Fundus. } In each case treatment with acid greatly |
| $53^2$ | + | + | — | Pylorus. } increased the activity of the extract. |
| $54^1$ | + | + | — | Calf. Fundus. |
| $54^2$ | + | + | — | " Pylorus. |
| XXI. | | | | XXI. Pig. |
| 74 | — | — | + | An old glycerin extract of uncertain age containing some HCl. |
| 75 | 0 | — | + | Glycerin extract, about 2 years old. |
| 78 | — | — | 0 | An acid (HCl, 0.2%) extract, about 3 years old. |
| $76^1$ | 0 | + | + | Fundus. |
| $76^2$ | 0 | + | + | Pylorus. |
| $77^1$ | 0 | + | + | Fundus. } Same results after four months, although |
| $77^2$ | 0 | — | + | Pylorus. } the odor of each extract was bad. |
| XXII. | | | | XXII. Dog. |
| 55 | 0 | + | + | Similar results were obtained with a longitudinal half of the mucous membrane exposed to air in a warm room for 5 hours before extraction. |
| 56*a* | 0 | + | + | *a* is the chloroform extract of a longitudinal half of the mucosa; *b* is a glycerin extract of the other half; *c* is a chloroform extract of the substance used for *b*. |
| 56*b* | 0 | + | 0 | |
| 56*c* | 0 | + | + | |
| 57*a* | 0 | + | + | |
| 57*b* | 0 | + | — | *b* was a fluoride extract which decomposed in 5 weeks and before it was tried with acid. |
| 58 | 0 | + | + | Stomach full. |
| 92*a* | 0 | + | + | |
| 92*b* | — | + | + | *b* was an HCl extract of half the stomach. |
| $59^1$ | 0 | + | [+] | Fundus. } Only tried with $CaCO_3$. |
| $59^2$ | 0 | 0 | [+] | Pylorus. } |
| $91^1$ | 0 | + | + | Fundus. |
| $91^2$ | 0 | + | 0 | Pylorus. Also 0 for calcium salts. |
| $93^1$ | 0 | + | + | Fundus. |
| $93^2$ | 0 | + | [+] | Pylorus. Best results with calcium salts. |
| XXIII. | | | | XXIII. Cat. |
| 60*a* | 0 | — | + | *a* is a chloroform extract of a longitudinal half of the mucosa; *b* is a glycerin extract of the other half; *c* is a chloroform extract of the substance extracted for *b*. |
| 60*b* | 0 | + | + | |
| 60*c* | 0 | + | + | |
| 61*a* | 0 | — | + | |
| 61*b* | 0 | + | 0 | 60*b*, after acidulation, was much less active than 60*c*. |
| 61*c* | 0 | + | + | |
| 62 | 0 | + | + | |
| 63 | 0 | + | + | + for acidulation during 3 days, but 0 when acidulated for 4 months. |
| 65 | 0 | 0 | [+] | 65. Stomach full. + for $CaCO_3$. |
| $64^1$ | 0 | + | + | Fundus. |
| $64^2$ | 0 | — | + | Pylorus. |

TABLE I.—*Continued.*

| No. | Plain Extract. | | Extr. + Ā. | Remarks. |
|---|---|---|---|---|
| | Milk. | Fibrin. | Milk. | |
| $66^1$ | 0 | + | + | Fundus. } Stomach full. |
| $66^2$ | — | + | [+] | Pylorus. } $66^2$ + only for calcium salts. |
| | | | | Kittens. |
| 67 | 0 | + | + | Age not noted. Acidulated 8 days. |
| 68 | 0 | trace | + | 1 week old. Acidulated 9 days. |
| 69 | 0 | + | + | 2–3 weeks old. Acidulated 6 days. |
| 70 | 0 | — | 0 | Born dead. Extract kept 170 days. Acidulated 1 and also 3 days. |
| 72 | 0 | 0 | 0 | Four together. One week old. Stomachs full. 0 also for calcium salts. |

TABLE II.

BESIDES THE OBSERVATIONS OF TABLE I A CERTAIN NUMBER WERE MADE ON MATERIAL PRESERVED IN STRONG (95%) ALCOHOL FOR VARIOUS LENGTHS OF TIME, AND THEN EXTRACTED WITH CHLOROFORM WATER. THE ESSENTIAL NOTES ARE AS FOLLOWS:

| No. | Time in alcohol. | Plain Extract. | | Extr. + Ā. | Remarks. |
|---|---|---|---|---|---|
| | | Milk. | Fibrin. | Milk. | |
| 4 | 24 d. | 0 | — | 0 | Skate. |
| 5 | 30 d. | 0 | 0 | 0 | Dog-fish. Also 0 for $CaCO_3$. |
| 9 | 28 d. | 0 | 0 | 0 | Cod-fish. Also 0 for $CaCO_3$. |
| $9a$ | 28 d. | 0 | — | 0 | " |
| 28 | 2 mos. | 0 | 0 | 0 | Gull. Also 0 for $CaCO_3$. |
| $53a^1$ | 14 d. | 0 | + | [+] | } Ox. Fundus and pylorus, part of the material |
| $55a^2$ | 14 d. | 0 | 0 | [+] | } used for $53^1$ and $53^2$ of Table I. Both acidulated *extracts* + for $CaCO_3$. |
| 71 | 5 mos. | 0 | 0 | 0 ? | Kittens. (Age ?). Stomachs full. Extraction of *substance* of mucosa with acetic acid gave an active extract when neutralized with $CaCO_3$. |
| 73 | 5 mos. | 0 | 0 | 0 ? | Kittens. (Age ?). Stomachs empty. + when substance is treated as in 71. |

An examination of Table I shows that the milk-curdling ferment was extracted by chloroform water in an active form, as pexin, from the stomachs of only two animals, an ox (53) and a calf (54), being present here in both the pyloric and fundal portions of the organ. In another ox (51) the extract was inactive, despite the fact that the extraction had lasted two weeks. The current view, based mainly on the researches of Hammarsten, is that an active ferment may be obtained from the stomach of the sheep as well as from that of the calf. The observations tabulated above were made on three sheep (48, 49, 50) and do not confirm this view. It is of course quite possible that a more extended series would give a different result, but it

is equally possible that a more extended study of the ox stomach would alter our views concerning that animal.

The table also contains memoranda of fifty-three different stomachs which gave up pexinogen to the extracting liquid, as shown by its behavior after treatment with a dilute acid. Of these fifty-three animals, thirty-three are mammals and twenty belong to other divisions of the vertebrates; absolutely negative results are noted for seventeen stomachs, and eleven of these cases belong to the mammalian group. There are fifteen stomachs recorded whose extracts gave positive results under special conditions which will be discussed below. There are also two stomachs whose classification is doubtful.

The absolutely negative cases are mostly scattered among positive experiments in the table, and a general explanation of them is not easily found. The mice (Nos. 39–43) are perhaps an exception. With regard to these it may be said that they were of course small, and it is therefore quite probable that the extracts were more dilute than usual. The extent to which the dilution of the ferment affects the curdling of milk has evidently not been fully worked out, and our attention was not directed to this danger until the experiments were practically finished. Later observations, as yet incomplete, have shown that the dilution of the ferment really does play a curious part in the process of coagulation. The same explanation may be offered for one of the very young rabbits (47), for the kittens numbered 70 and 72, and perhaps for the snake (32).

The condition of the stomachs is noted, most unfortunately, for only twenty-two of the eighty-seven stomachs under discussion. For five the record reads "empty," so that seventeen contained more or less food. Thirteen of these are among the stomachs which gave absolutely negative results or where the existence of the pexinogen was only shown by the special treatment. It may therefore be inferred that an extract from a gastric mucous membrane which has been secreting freely for purposes of digestion is not as rich in pexinogen as that from a resting membrane.

The acidulated extracts were commonly neutralized by adding carbonate or bicarbonate of sodium in substance. The determination of

the exact point of neutralization by means of the ordinary indicators presented some difficulties, and these were not lessened by the experience that some of our extracts were unexpectedly sensitive to alkalies. We tried also neutralization by means of calcium carbonate. This is theoretically a simple process, but it was found to be much more uncertain with extracts containing acetic acid than when hydrochloric acid has been used. The circumstance that we should thus increase the calcium salts in solution seemed to be an additional advantage, since many investigators have found such a procedure helpful. Calcium carbonate was also tried in a number of cases where neutralization with sodium carbonate had given negative results, and in some the addition of calcium chloride was also tried. Here belong the experiments mentioned above as showing pexin under special conditions of treatment. Concerning these it may be said that many extracts which, despite treatment with acid for a suitable time, do not coagulate milk, will do so when certain amounts of calcium compounds are added. Something like this has been noticed by others, but has generally been explained as due to a deficiency of calcium in the milk. The observations of this paper do not give us material for any final opinion on this subject. Taken in connection with some other unfinished experiments they do, however, enable us to say tentatively that the deficiency of calcium in these cases is not necessarily a defect of the milk which happens to be used, but stands probably in some relation to the amount of the ferment and perhaps also to the chemical reaction of the solution. In addition to a few cases where only calcium carbonate was used there are twelve extracts which were negative when neutralized with sodium carbonate, but positive when calcium carbonate was employed.

The experiments with absolutely negative results call for a brief comment. Five of them were tested only with sodium carbonate, and the remaining twelve were negative despite a possibly insufficient addition of calcium carbonate. The dilution of the extracts, already suggested as a partial explanation of certain cases, can hardly be the cause of failure in the case of some of the rats (90d, 90g) and kittens (70, 72), since other stomachs in the same groups and of equal size

and fulness gave positive results. The failure of the pig extract (78) may be due to its age and to its acidity, although some of our experiences have made us sceptical as to the accepted view that the curdling ferment is rapidly destroyed by the digestive juices. Striking, too, is the fact that not one of the dog-fish yielded an active extract, while positive results were common for the related skate and the mackerel-shark. We shall look into this question again when we can get the material.

We have made a limited use of glycerin as an extracting and preserving medium. Our results are somewhat discordant. The memoranda concerning the dog (56) and the two cats (60, 61) show that glycerin does not extract the pexinogen from these stomachs as readily as chloroform water does. Both the glycerin extracts from the pig (74, 75) contained the curdling ferment. These extracts were old and of somewhat uncertain origin, so that much importance ought not to be attached to them. Recent study of the stomach of the pig makes it probable that glycerin does extract the zymogen from it with considerable ease.

As a control we have used water for extracting the ferment in a few cases which do not appear in the table. Such extracts became putrid, of course, in a few days and thus enabled us to make a small number of observations concerning the action of putrefaction in the pexinogen. The change in odor was taken to be indicative of putrefaction. Our memoranda refer to aqueous extracts of parts of stomachs of two sheep (49, fundus and pylorus; 50, pylorus) and two pigs (76, fundus and pylorus; 77, fundus), other portions of the same stomachs having been used for chloroform extracts. All six extracts were still inactive at the end of twenty-four hours, and two (77 and 50) were not tested further. One (49, pylorus) remained negative for several days, although malodorous, while the fundus extract of the same sheep was active on the third day, although putrefaction was not otherwise noticeable until the fifth or sixth day. Both the water extracts of the other pig (76) had become active after five days and also offensive. The number of observations is small, but the conclusion seems warranted that putrefaction, at least in its

earlier stages, sets the ferment free. This view is favored, too, by the experience that chloroform extracts become active when putrefaction sets in, as appears in the notes of the table for one of the turtles (24a) and for two of the sheep (49, 50). This influence of putrefactive changes is of importance from another point of view. It has doubtless attracted the attention of the reader that the early investigators, Spallanzani and Hunter, found active extracts in many instances without any acidulation or other special treatment, while such extracts are the exception in the researches of Hammarsten and those noted in this paper. It might be urged that the presence of chloroform has in some way modified our results, but, for reasons already sufficiently detailed, we do not believe that to be the case, and certainly no such objection can be urged against the observations of Hammarsten. If it be assumed that the extracts of Spallanzani and Hunter had undergone some slight putrefactive changes, too slight perhaps to attract much attention, we can then understand how these authors found their extracts so frequently active. It should also be borne in mind that their records seem to contain only the positive results, perhaps merely generalizations, and certainly without any detailed account of the experiments.

Table II giving results obtained where the material has been preserved in alcohol presents some points of interest. The dogfish may be at once excluded, since equally negative results were found without alcohol. For the rest it may be noted that the most efficient extracts were those where the action of the alcohol had lasted the shortest time. We also tried the effect of alcohol on active chloroform extracts and found the precipitate still active after seventeen days' exposure to the alcohol. It is evident that the influence of alcohol on the ferments in the mucous membrane itself needs further study.*

The records of this paper relate only to the gastric mucous membrane of vertebrates. We have also made extracts from the alimentary

* The irregularity in the action of alcohol has attracted the attention of other investigators. Cf. p. 70 of Podwyssozki's article: Zur Methodik der Darstellung von Pepsinextracten. Pflüger's *Arch.*, xxxix, 62.

tract of the lobster and have satisfied ourselves that it too sometimes gives up to chloroform water a substance which, when acted upon by an acid and then made neutral with calcium carbonate, coagulates milk. It is therefore probable that pexin is not confined to the vertebrate digestive apparatus, and we hope to be able to search further for it among invertebrates. That a milk-curdling ferment exists in the juices of many plants is already well known.

# NOTE ON THE INFLUENCE OF "PEPTONE" ON THE CLOTTING OF MILK BY RENNET.

By F. S. LOCKE, M. A. (Cantab.), M. B. (Lond.).

E. Gley* and A. Edmunds† have recently independently shown that Witte's "peptone" has a distinct effect in delaying the clotting of milk by rennet. An abstract of Gley's paper in the *Centralblatt für Physiologie* has made me aware (January, 1897) of the results of these workers. In October and November, 1894, I made experiments to determine the effect of "peptone" on the action of rennet on milk, and will shortly state the results of them here, as they differ in some not unimportant respects from those already published.

Gley and Edmunds both used Witte's "peptone" in their experiments.‡ I, at first, also made use of this, and obtained with it results exactly comparable with those of these observers. On testing the reaction of aqueous solution of Witte's "peptone," however, I found it markedly alkaline to red litmus paper. As alkalies have themselves a marked influence in delaying and even preventing the action of rennet on milk,§ the results I had obtained appeared to me to be valueless, and it became a question to obtain a "peptone" neutral or faintly acid in reaction.

Schuchardt's "peptone" I found to resemble Witte's in its action on red litmus. It was, therefore, useless for my purpose.

* E. Gley, *Compt. rend. de la Soc. de Biol.*, 1896, 591.

† A. Edmunds, *Journ. of Physiol.*, xix, 474, 1896.

‡ With the exception of one experiment reported by Gley in which 5 cc. of milk mixed with 3 cc. of a 10 per cent aqueous solution of "propeptone," made in accordance with the description of Grosjean, were acted on by rennet. The reaction of the propeptone is not mentioned. Judging from a statement of Spitzer's (*Arch. f. d. ges. Physiol.*, l, 573, 1891) it would probably have been alkaline to lacmoid.

§ See p. 496 of this paper.

Gehe's "peptone," however, I found to be amphoteric in reaction, turning red litmus paper faintly blue and blue litmus paper faintly red. Its aqueous solution gave almost immediately a very distinct precipitate when an alkaline oxalate was added, pointing to the presence of soluble calcium compounds. It had the same action as Witte's "peptone" in delaying the action of rennet.*

Grübler's "peptone" was next made use of. Its aqueous solution was markedly acid to blue litmus paper and did not turn red litmus paper blue. It gave no precipitate in some minutes with ammonium oxalate and ammonium hydrate; only traces of calcium could therefore be present in it. Its effect in delaying the action of rennet was quite

* Experiment, November 3, 1894. The rennet used was, as in all my experiments, Crosse and Blackwell's, and was neutral in reaction to litmus. The "peptone-milk" was made by dissolving 1 gramme of Gehe's "peptone" in milk, and diluting with milk to 100 cc.

| Time p. m. | Remarks. | 10 cc. milk + 0.1 cc. 10% $CaCl_2$. | 10 cc. milk. | 10 cc. 1% peptone-milk + 0.1 cc. 10% $CaCl_2$. | 10 cc. 1% peptone-milk. |
|---|---|---|---|---|---|
| 3.21–22 | 0.3 cc. rennet added to each. | | | | |
| 3.22 | The four tubes placed in water-bath at 39° C. | | | | |
| 3.25 | | Thickening. | | | |
| 3.25¼ | | Pours with difficulty. | | | |
| 3.26 | | Clotting complete. | | | |
| 3.28½ | | | Pours with difficulty. | | |
| 3.28¾ | | | Clotting complete. | | |
| 3.30 | | | | Thickening. | |
| 3.30½ | | | | Pours with difficulty. | |
| 3.31½ | | | | Clotting complete. | |
| 3.35 | | | | | Thickening. |
| 3.37 | | | | | Pours with difficulty. |
| 3.40 | | | | | Clotting complete. |

marked,* although not so much so as that of Gehe's "peptone," a difference very likely due to their difference in reaction, since acidity favors the action of rennet.

After these results it might appear that the reaction of "peptone" in delaying the clotting of milk by rennet was sufficiently established as being independent of any change in reaction caused by its addition, more especially since Gehe's "peptone" contained easily detectable soluble calcium compounds, which would themselves tend to hasten the action of rennet.† This is not, however, I think, the case. Our conceptions of "neutrality," "acidity" and "alkalinity" have, in recent years, undergone very great modifications, owing to the discovery of new indicators which differ in their behavior with the same chemical compounds.‡ Grübler's "peptone" affords an example of this, for

* Experiment, November 6, 1894.

| Time p. m. | Remarks. | 9 cc. milk +1 cc. $H_2O$. | 9 cc. milk +1 cc. $H_2O$. | 9 cc. milk +1 cc. 10% $H_2O$ solution of Grübler's "peptone." | 9 cc. milk +1 cc. 10% $H_2O$ solution of Grübler's "peptone." |
|---|---|---|---|---|---|
| 3.35–37 | 0.5 cc. rennet added to each. | | | | |
| 3.38½ | The four tubes placed in water-bath at 41° C. | | | | |
| 3.40 | | Flakes. | Flakes. | | |
| 3.43 | | Thickening. | Thickening. | | |
| 3.43½ | | Pours with difficulty. | Pours with difficulty. | | |
| 3.44 | | Clotting complete. | | | |
| 3.44½ | | | Clotting complete. | | |
| 3.46½ | | | | | Thickening. |
| 3.47 | | | | Thickening. | Pours with difficulty. |
| 3.48 | | | | Pours with difficulty. | |
| 3.50 | | | | Clotting complete. | Clotting complete. |

† See experiment in foot-note * on p. 494. This effect of calcium salts has been observed by Hammarsten and many other workers.

‡ See R. M. Thomson, *Chem. News*, xlvii, 123 and 184, 1883; xlix, 32, 38, and 119, 1884; lii, 18 and 29, 1885. A. H. Allen, *Pharm. Journ. and Trans.*, xxii (3rd series), 752 and 772, 1892, gives a good general account of the subject. G. Courant's valuable paper, über die Reaction der Kuh- und Frauenmilch (*Arch. f. d. ges. Physiol.*, l, 108, 1891), treats of it especially in relation to milk.

although markedly acid to litmus, it was markedly alkaline to lacmoid, as was also Gehe's "peptone," which to litmus reacted amphoterically. These substances must, therefore, contain salts of "weak" acids, or bodies acting as such, which are possibly the cause of their power to delay clotting. This possibility prevented the publication of my experiments.

That increase in the alkalinity of milk hinders its clotting by rennet was pointed out in 1872 by Soxhlet,* Heintz† and Hammarsten.‡ Soxhlet and Heintz found that small additions of sodium carbonate, Hammarsten§ of sodium hydrate, had this effect.

Duclaux‖ has investigated the influence of various substances on the action of rennet and finds that sodium carbonate, sodium borate, borax, and even boric acid, delay it. He observed also that sodium and calcium hydrates have the same effect.

Söldner¶ found that the coagulability of milk by rennet disappeared when it was rendered no longer acid to phenolphthalein by sodium hydrate. Courant** found that lime water exerts a similar action.

Ringer†† observed that a small percentage of sodium bicarbonate prevents the action of rennet. Arthus and Pagès‡‡ found that while sodium carbonate had this effect, sodium bicarbonate apparently had not.

* F. Soxhlet, zur physiologischen Chemie der Milch. Dissertation, Leipzig, 1872 (*Journ. f. prakt. Chemie*, N. F., vi, i, 1872).

† W. Heintz, *Journ. f. prakt. Chemie*, vi (N. F.), 374, 1872.

‡ O. Hammarsten, *Upsala Läkareförenings Förhandlingar*, viii, 63, 1872. This paper I have been unable to consult in the original. It is abstracted by Hammarsten himself in *Jahresber. ü. d. Fortschr. d. Thierchemie*, ii, 118, 1874, and by Panum in *Jahresber. ü. d. Fortschr. d. Anat. u. Physiol.*, ii, 392, 1875.

§ Hammarsten found that "certain salts, especially sodium phosphate (not only neutral, but also an amphoterically reacting mixture of neutral and acid sodium phosphate), delay or prevent coagulation by rennet." (*Jahresber. ü. d. Fortschr. d. Anat. u. Physiol.*, ii, 392, 1875.) What percentage of these salts Hammarsten worked with I have not been able to find.

‖ E. Duclaux, *Annales de l'Institut Agronomique*, iv, 1880 (this paper I have been unable to consult), Encyclopédie Chimique, ix (1), 177, Paris, 1883, and Le Lait, Paris, 1887, p. 109.

¶ F. Söldner, die Salze der Milch. Dissertation, Erlangen, 1888 (*die landwirthschaftliche Versuchstationen*, xxxv, 351, 1888), pp. 78 and 84.

** G. Courant, op. cit., p. 146.

†† S. Ringer, *Journ. of Physiol.*, xi, 474, 1890.

‡‡ M. Arthus and C. Pagès, *Arch. de Physiol.*, xxii, 336, 1890.

Söldner endeavored to account for the favorable influence of acids for rennet and the unfavorable influence of alkalies by their action on the calcium salts in milk, acids increasing the amount of soluble calcium salts at the expense of the insoluble suspended tricalcic phosphate normally present, and alkalies having the reverse effect. If alkalies act only in this way it is difficult to see how Gehe's "peptone," containing as it does soluble calcium salts, can act as an alkali when added to milk and precipitate these. Söldner himself, however, refuses to deny that the degree of alkalinity may exercise a specific influence apart from its effect on the amount of calcium salts in solution, and from the evidence hitherto obtained I hesitate to draw any conclusion as to whether "peptone" has any specific effect on the coagulation of milk by rennet.

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Grosjean,* who investigated in Fredericq's laboratory the influence of peptone and propeptone on the coagulation of the blood both when injected intravascularly and when mixed with it shed, declares that propeptone must be added in so great a proportion to shed blood to delay or suppress coagulation that its action cannot be regarded as different from that of strong solutions of neutral salts, of sugar, and of other indifferent substances.

Such an explanation of the action of "peptone" is, however, justifiable neither in the case of blood nor of milk. Grosjean† in one experiment lengthened the time before coagulation began from three to fourteen minutes, and the time before complete coagulation took place from eight to thirty-four minutes, by the addition of 2.5 per cent of "propeptone." Pollitzer‡ before him had found 2 per cent of heteroalbumose to increase the time necessary for coagulation from between 3 and 5 minutes to between 15 minutes and 24 hours. Edmunds and I observed, respectively, a distinct effect in delaying the clotting of

* A. Grosjean, *Memoires couronnes et autres Memoires publiés par l'Académie royale de Belgique*, xlvi, 1–80, 1892 (somewhat condensed in *Arch. de Biol.*, xii, 381, 1892, and *Travaux du Laboratoire de L. Fredericq*, iv, 45, 1892).

† Op. cit., p. 28.

‡ S. Pollitzer, *Journ. of Physiol.*, vii, 288, 1886.

milk of 0.625 per cent and 1 per cent of "peptone." Now Hammarsten* found that 0.25 per cent of sodium chloride and of potassium chloride had no effect on the "Gerinnungszeit" of milk, and that 1 per cent of sodium chloride from three and a half minutes only increased it to four minutes, and 1 per cent of potassium chloride from two minutes only increased it to two and a half minutes. When the high molecular weights of albumoses† (relatively to these salts) is taken into consideration, it is impossible to assume that their action is analogous to that of neutral salts.

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Gley, Edmunds and myself noticed that the effect of "peptone" in delaying the action of rennet can be antagonized by calcium chloride. I found afterwards, as had already been established by several observers, that calcium chloride added to milk in the absence of "peptone" increased the activity of rennet. Its action, therefore, in the presence of peptone may consist not in directly antagonizing an unfavorable influence, but in exercising independently a favorable one, and can be in no wise regarded as a support of the view that the action of "peptone" consists essentially in an attraction for calcium, which has been insisted on especially by Pekelharing‡ as the explanation of the action of "peptone" on the coagulation of the blood. Such a power to fix calcium in solution is very difficult, if not impossible, to conceive, and Pekelharing himself states that "peptone" gives no precipitate with soluble calcium salts. Just too as calcium chloride added to normal milk favors its coagulation by rennet, so does the intravenous injection of calcium chloride into the normal blood hasten its coagulation when shed, as Wright§ and others have found, a result which I also obtained without knowledge of their work in unpublished experiments on cats made in consequence of the experiments I had

* O. Hammarsten, zur Kenntniss des Caseins und der Wirkung des Labferments, Upsala, 1877, pp. 70 and 71.

† See Hammarsten, Lehrbuch der physiologischen Chemie, 3te Auflage, Wiesbaden, 1895, pp. 32 and 634.

‡ C. A. Pekelharing, Virchow's Festschrift, i, 433, 1891.

§ A. E. Wright, *Brit. Med. Journ.*, 1891 (ii), 1306.

made on milk. In the case, then, of the coagulation of the blood also, the annulling of the effect of "peptone" by calcium chloride may be due not to its directly undoing the work of "peptone," but to the same action favorable to extravascular coagulation which it exerts on unpeptonized intravascular blood.

It may be added that Halliburton and Brodie concluded merely from the fact that calcium chloride injected intravenously annulled the well-known effect of Haycraft's leech-extract in doing away with the coagulability of the blood, that this substance owes its power, like "peptone," to an "affinity" for calcium.*

Harvard Medical School, Boston, Mass., *April*, 1897.

* W. D. Halliburton and T. G. Brodie, *Journ. of Physiol.*, xvii, 147 and 160, 1894.

# A STUDY OF THE VISCERAL CHANGES IN EXTENSIVE SUPERFICIAL BURNS.

By CHARLES R. BARDEEN.
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PLATES XXXVI AND XXXVII.

Many hypotheses have been brought forward to account for the constitutional symptoms following extensive superficial burns. Few of these rest upon a physiological and anatomical basis sufficient to make them of enduring value.

Theories as to the general pathology of cutaneous burns have been almost as numerous as the writers of articles on the subject. The more elaborate are the following:

I. Theories involving the idea that death after burns is due to loss of normal cutaneous activity, especially as regards—

a. The respiratory function, leading to overwork in the lungs (Heyfelder and others) or asphyxia (Bouley). Yet the skin performs less than one twenty-fifth part of the total normal respiration of the body.

b. The perspiration, causing overwork of the abdominal organs (the earlier writers) or retention of poisonous organic substances (Billroth*). Billroth accepted this idea on the basis of the conclusions drawn by Edenhuizen from experiments in "gilding" animals. More recent work on this latter subject tends to show, however, that death following coating with impervious substances the skin of animals is to be attributed rather to the absorption of products of altered metabolism in the skin than to the retention of normal excretory products (Kijanitzin†).

c. The heat-regulating functions (Laschkewitsch,‡ Falk§). But it has been shown that death with lowering of the body temperature may follow burns even where loss of heat by radiation is carefully guarded against by coverings.

* *Arch. f. klin. Chirurgie* (1865), vi, 406-408.
† Virchow's *Archiv* (1893), cxxxi, 436-467.
‡ *Arch. f. Anat. Phys. u. wissen. Med.*, 1868, 61.
§ Virchow's *Archiv* (1871), liii, 27-69.

d. The protective functions. This may account for some deaths due to secondary bacterial invasion.

II. Theories involving the idea that death is due to excitation of the nervous system, and consequently to—

a. Shock. Death is wont to be attributed to the effects of shock when no pathological alterations sufficient to account for a fatal result are found at autopsy.

b. Reflex exhaustion and loss of vascular tonus (Sonnenburg,* Tschmarke†). The experiments of Sonnenburg, on which this theory was based, have been offset by those of others who have shown that death ensues as rapidly when the nervous connections with the burned areas (for instance, a rabbit's ear) are cut as when they are left intact (Salvioli,‡ St. Markusfeld and Steinhaus.§) That there is some vaso-dilatation due to nervous exhaustion seems probable (Boyer and Guinard‖).

III. Theories involving the idea that death is due to heart paralysis caused by—

a. Cooled blood (Falk¶). Falk supposed, further, that the cutaneous vessels are so dilated by the direct action of the heat that the heart is deprived of sufficient blood to beat against.

b. Overheated blood (Sonnenburg**).

c. Poisonous substances in the blood (Boyer and Guinard††).

IV. Theories attributing death to alterations in the circulation due to—

a. The blood being "driven in" by the heat (earlier writers).

b. The accumulation of the blood in the peripheral vessels dilated by the burn (Falk‡‡).

c. Thrombosis (Silbermann,§§ Salvioli,‖‖ Welti,¶¶ St. Markusfeld and Steinhaus***).

* *Deutsche Ztschrft. f. Chirurgie*, (1878), ix, 138-159.
† *Deutsche Zeitschrift f. Chirurg.*, (1896), xliv, 346-392.
‡ Virchow's *Archiv*, 1891, cxxv, 364-397.
§ *Centralblatt f. allg. Path. u. path. Anat.* (1895), vi, 1.
‖ Des Brûlures, Paris, 1895.
¶ Loc. cit. ** Loc. cit. †† Loc. cit. ‡‡ Loc. cit.
§§ Virchow's *Archiv* (1890), cxix, 488-523.
‖‖ Loc. cit.
¶¶ *Centralblatt f. allg. Path.* (1890), i, 537-550.
*** Loc. cit.

V. Theories involving the idea that alteration in the blood causes death through—

a. Destruction of red blood corpuscles (Max Schulze,* Werthheim,† Ponfick‡).

b. Loss of function in the red blood corpuscles (v. Lesser§).

VI. Theories involving the idea that death is due to the presence of poisonous substances in the blood produced by—

a. Increase in fibrin ferment (Foà).

b. Formation of hydrocyanic acid in the skin (Catiano||).

c. Potassium salts set free by the destroyed red blood corpuscles (Schjerning¶).

d. Alteration in metabolism or tissues (Kijanitzin,** Ajello and Parascandolo††).

e. Absorption of the products of bacterial activity from the burned areas (Lustgarten‡‡).

The clinical picture presented after extensive burns of the skin is a striking one. The unfortunate victim, after a brief period of excitement and agony caused by extreme pain, soon loses the power of much movement or resistance. The intelligence, at first fairly clear in spite of the great shock, gradually becomes beclouded. At times there may be delirium and cramps. The skin in the burned areas usually rapidly undergoes œdematous swelling. The pulse becomes very weak and rapid. A soft systolic murmur may often be heard over the heart. The respiration becomes rapid, irregular, and shallow. The temperature, at first rising quickly above the normal, soon becomes subnormal in rapidly fatal cases. There may be vomiting and diarrhœa. The urine often shows traces of hæmoglobin and albumin. Death within forty-eight hours is very apt to ensue if two-thirds of

* *Arch. f. mikr. Anat.* (1865), i, 1.
† *Wien. med. Presse* (1867), viii, 1237-1239 et seq.
‡ *Berliner klin. Wochenschrift*, 1876, No. 17; 1877, No. 47.
§ Virchow's *Archiv* (1880), lxxix, 248-310.
|| Virchow's *Archiv* (1882), lxxxvii, 345-364.
¶ *Vierteljahrschr. f. gericht. Med.*, N. F., xli, xlii (1884-5).
** Virchow's *Archiv* (1893), cxxxi, 436.
†† *Gazzetta degli ospedali e delle cliniche*, Miliano (1896), xvii, 866-872.
‡‡ *Wien. klin. Wchnschft.*, 1891, No. 29, 528-531.

the surface of the body is involved, even though the burn locally has produced little more than an erythema.

At autopsy, if death has occurred very soon after the burn, there may be no internal pathological lesions noted. Or there may be seen venous congestion of the serous membranes and of the viscera. This hyperæmia is likely to be more marked in different regions in different cases. Occasionally ecchymoses occur in the serous membranes. In those cases where death has not ensued for some hours the kidneys and liver may appear quite cloudy; while if the patient has lived for several days there may be well marked inflammation of the intestinal canal, the lungs, pleura, kidneys, and meninges. Occasionally gastric or duodenal ulcers are seen.*

It has been definitely shown both by experimental work on animals and by clinical investigation that the blood is markedly altered after superficial burns. The better established of the changes said to take place in the blood are the following:

1. The specific gravity is slightly raised (Tappeiner†).

2. The erythrocytes are in part injured morphologically (Wertheim‡) and functionally (v. Lesser,§ Silbermann||).

3. There is a marked leucocytosis, chiefly of polymorphonuclear cells.

The following effects of the changed blood on the body seem to have been proven:

1. Fragments of red blood corpuscles are collected in cells of the spleen, bone-marrow and lymphatic glands; blood pigment is seen in the epithelium of the kidneys.

2. Thrombosis occurs at times in the capillaries and veins of some of the internal organs.

The majority of the text-books of to-day attribute death following burns to thrombosis. But recently there has again come into view the idea that after burns there come into existence and circulate in the

* For a very complete account of the autopsy reports of cases of skin burn published before 1883, see Schjerning, *Vierteljahrschr. f. gericht. Med.*, N. F., xli, xlii (1884-85).

† *Centralbl. f. d. med. Wissenschaft.*, 1881, 385, 401.

‡ Loc. cit. § Loc. cit. || Loc. cit.

blood toxic substances which give rise to the constitutional symptoms spoken of above. At one time it was thought that loss of cutaneous activity led to the retention in the blood of noxious substances normally excreted through the skin. But no very poisonous substances have been found in the cutaneous secretions. Those holding theories of a toxæmia after burns to-day assume that altered conditions in the affected areas give rise either to the poisonous substances or to substances which are absorbed and cause the internal organs to produce toxic substances.

Unfortunately, definite proof of the existence of these substances has not yet been offered. Kijanitzin,* by a modification of Brieger's method, obtained from the blood of variously burned dogs substances which he says are much like the Brieger peptotoxins. Ajello and Parascandolo† convinced themselves, though the published account of their work fails to convince others, that burned tissues are rich in toxic substances. Boyer and Guinard,‡ E. Spiegler§ and A. D. Pawlowsky‖ all failed to obtain definite toxic substances from the blood or tissues of burned animals. Pawlowsky denies their existence, while Spiegler and Boyer and Guinard consider that toxic substances do circulate in the blood after superficial burns, but in a state not to be detected by known chemical methods. The endeavor to obtain toxic substances from the urine in these cases has proved equally unsatisfactory, though some investigators have found the urine of patients and animals suffering from burns of increased toxicity to rabbits.¶

Much fruitful study has been made in recent years of the changes in the tissues following alterations experimentally produced in the blood and the circulation of animals. The facts thus gained make it possible to refer many lesions found in the human body after death to fairly definite antecedent conditions of the blood-supply to the parts affected. These observations render of additional value a careful

* Loc. cit. † Loc. cit.

‡ Des Brûlures, Paris, 1895.

§ *Wien. med. Bl.* (1896), xix, 359, 277, 294, 310.

‖ Atti d. XI Cong. Med. Internaz. (1894), Roma, 1895, iv Chirurg., 267-269.

¶ Reiss, *Wiener med. Wchnschrft.* (1893), xliii, 59-61.

study of the organs after death in cases where it may be suspected that the blood has been altered either in quantity or quality. This is especially true of cases of cutaneous burn.

The opportunity has recently been offered the writer of making a systematic study of the organs from the bodies of five children fatally burned. The children varied in age from sixteen months to eight years. The time elapsing between the burn and death varied from four to nine and one-half hours. The extent of the burns superficially as well as the lesions found in the internal organs were so strikingly alike in all cases that it has been deemed unnecessary here to give a detailed account of each case.

Four of the autopsies were made and described by Dr. Flexner, the other by Dr. George Blumer; all were held very soon after death.

*Post-mortem appearances.*—Exterior. Extensive burns covering pretty much the whole body and of varying degrees of intensity in different regions. While chiefly of the first and second degrees, in places the lesions extended so deeply that the superficial layers of the skin peeled off and the deeper layers were so altered as to be very friable. Oedema was very marked, especially about the face and eyelids. In no case was there charring, nor were there macroscopical evidences of the burn below the œdematous subcutaneous tissue. But few cutaneous ecchymoses were seen.

The muscles appeared normal; the axillary and inguinal lymphatic glands were enlarged and soft.

*Thoracic cavity.*—On opening the thoracic cavity the pleuræ appeared normal, the lungs slightly congested and œdematous, and the larger bronchi congested. The pericardial sac was normal; likewise the heart, save pallor of the muscle. The thymus was congested; the cervical lymphatic glands swollen and more or less congested.

*Abdominal cavity.*—On inspecting the abdominal cavity no excess of fluid was seen. The intestines were moderately distended; the visceral peritoneum was slightly congested. The liver on section was pale and cloudy. The spleen was enlarged and soft, with rather prominent Malpighian bodies. The kidneys were pale; glomeruli not prominent, cortex cloudy. Adrenals and pancreas were normal. The stomach and intestines showed swelling of the solitary and agminated follicles throughout. These enlarged lymphatic patches were often surrounded by a slightly hyperæmic zone. The abdominal lymphatic glands were all greatly

swollen and some were congested. The genital organs showed nothing important.

*Cerebral cavity.*—Meninges not particularly congested. Brain negative. Cord not examined.

Cultures for bacteria proved negative, except that in one case the micrococcus lanceolatus was found in the cultures from the lungs.

To sum up: the chief gross morbid changes noted were cloudy swelling of the liver and kidney; softened, enlarged spleen; and above all, swelling of the lymphatic glands and of the gastro-intestinal lymph follicles. The hyperæmia of the thoracic and abdominal organs was moderate.

Microscopical examination of fresh teased tissues was made in the case of the spleen, liver and kidney; and in addition the blood was examined in the fresh state. The most striking thing noticed in these tissues was a considerable fragmentation of the red blood corpuscles.

Frozen sections of kidney, liver, spleen and heart-muscle were examined. No abnormal fat was seen. The liver and kidneys showed cloudy swelling. Many cells containing fragments of red blood corpuscles were seen in the spleen, and fewer in the liver.

Parts of the chief thoracic and abdominal viscera and, in one case, the brain were preserved for microscopic study.

The chief microscopic changes noted were as follows:

The skin where much burned showed heat-coagulation and the running together of the tissue elements of the upper layers. The subcutaneous tissues of the burned region and the lower layers of the corium were very œdematous. The smaller blood-vessels just under the epithelium were filled with destroyed blood and occluded by thrombi. The larger, deeper vessels of the skin and subcutaneous tissues contained, on the other hand, few thrombi. In two cases the epithelium of the sweat glands showed protoplasmic and nuclear degeneration (fragmentation). The skin of the scalp was affected in the same manner as the skin of the surface generally

The blood showed considerable poikilocytosis, and there was a marked leucocytosis, chiefly polymorphonuclear, as well. Thrombi,

excepting in the superficial layers of the skin, were not common. The bone-marrow showed great reproductive activity, though in areas foci of degeneration were to be discerned, and throughout were scattered cells containing fragments of red blood corpuscles.

The pulmonary tissue showed, besides congestion, in one case the early stages of a pneumonia. In this case the micrococcus lanceolatus was cultivated at autopsy.

The cardiac muscle appeared normal.

The liver showed parenchymatous swelling of the epithelium, with focal areas in which there was marked vacuolization of the nuclei and some, although slight, fragmentation. No hyaline degeneration was seen. Capillary thrombosis was, however, not very infrequent, and in several places karyokinesis was discerned in the capillary endothelium. A moderate congestion existed.

The splenic tissue showed congestion and much fragmentation of erythrocytes, many fragments being contained in phagocytic cells, many lying free. The Malpighian bodies showed focal degeneration, with nuclear fragmentation.

The chief lesions seen in the stomach and intestine were swelling of the lymphatic tissue (Plate XXXVI, Fig. 1), and in this focal necroses containing nuclear fragments. There was also increased activity in the goblet cells of the mucosa.

The kidneys showed very marked parenchymatous swelling of the convoluted and ascending tubules, much nuclear fragmentation in their cells, hæmorrhage into the glomerular capsules and from the capillaries between the tubules; blood pigment appeared in Henle's tubules and to a less marked extent in some other regions. Occasionally an occluded thrombosed vessel was found.

The examination of the sections from the brain revealed nothing of note. The same may be said of the adrenals, thyroid, thymus, and pancreas.

To sum up: the microscopic lesions of chief importance were the parenchymatous degenerations found in the liver, kidneys, and lymphatic tissues.

The lesions found in the lymphatic tissues were most interesting for

several reasons: their wide distribution throughout the body, their severity in spite of the few hours elapsing between the burn and death, and their specific and focal nature. Oedema and often focal areas of necrosis were found in the nodular collections of lymph cells throughout the body, from those in Glisson's capsule in the liver to the largest lymphatic glands. The lesions were so essentially alike that a detailed description will here be given merely of the changes noted in the lymphatic glands. In addition, brief reference will be made to the changes found in the lymphatic tissues elsewhere.

The lymphatic glands from the various cases and from the various parts of the body were affected in different degrees both microscopically and macroscopically. The minuter changes corresponded in the main with the gross appearances. The study of sections from various glands led to the conviction that the different steps in the pathological process could be followed.

A general œdema throughout the lymphatic gland, but especially marked at the germinal centres of the lymph follicles, seems to be the earliest stage of the process. This œdema is accompanied sometimes by slight venous and capillary congestion. It is at this stage, if at any, that there is an increased activity in cell multiplication. At this stage a few karyokinetic figures can usually be made out; in the later stages they are rarely seen.

Coincident with the œdema, the lymph cells, especially those at the germinal centre, begin to swell and to assume various distorted shapes. These swollen cells are quite different in general morphology from the proliferating cells usually found at the germinal centre. The protoplasm becomes coarse and granular, sometimes vacuolated; and the nucleus, at first vesicular, later takes on the form of knotted irregular bands of chromatin. Soon the cells go completely to pieces, scattering about small and large bits of a chromatin material which stains deeply in the basic anilin dyes. No fat, hyaline or fibrin was seen in these areas, in sections especially stained for their demonstration.

This necrosis starting at the centre of the lymph follicle extends towards the periphery, while the whole follicle becomes greatly en-

larged. In stained sections one can see with the naked eye the swollen follicles with their dark rim composed of normal lymphocytes and their much lighter centre where the degeneration is going on (Plate XXXVI, Fig. 2, and Plate XXXVII). Meanwhile the debris from the destroyed lymphocytes is rapidly cleared from the centre of the follicle, disclosing large flat endothelial cells, containing thin, faintly staining nuclei. If, as seems to me probable, there are lymph spaces, running throughout the follicle, lined by flattened endothelial cells, these large cells just spoken of represent such cells in a greatly swollen condition. At any rate they are great phagocytes and engulf the broken cells and nuclei.

The general appearance, therefore, of one of the more marked areas of degeneration in the lymph follicle is as follows (Plate XXXVI, Fig. 2, and Plate XXXVII): At the centre are large flat endothelial cells with granular protoplasm more or less filled with debris and containing a faintly staining nucleus. Scattered about are lymph cells undergoing various degrees of degeneration and necrosis. Outside of this central area comes a broad zone where extreme cellular necrosis is going on. Most varied pictures of distorted cells and nuclei, bits of chromatin and masses of disintegrating protoplasm are seen. Then comes an area marked chiefly by the presence of swollen leucocytes, and at the periphery nearly normal lymphocytes are packed together in a narrow rim. At times, however, the process of degeneration in the follicle is rather more diffuse, so that affected and apparently unaffected cells are more intermingled. In these cases concentric areas representing various degrees in lymphocytic necrosis are not clearly marked. The capillary walls are intact, so far as can be seen. There are, however, a few red blood corpuscles free outside the blood-vessels.

In the lymph cords degenerating cells may be seen scattered rather diffusely among normal-looking lymphocytes.

The swollen lymph sinuses contain besides great numbers of small mononuclear cells many large and transitional forms. In some instances a considerable number of polymorphonuclear leucocytes and of eosinophiles are seen. Both dividing and fragmenting lymphocytes also appear here and there.

The swelling of the glands is to be ascribed almost entirely, if not wholly, to the œdema, to the swelling of individual cells, and to the extensive fragmentation of the latter. In a given area of a swollen gland there are fewer cells than in one of a corresponding size in an unaffected gland.

In the Malpighian bodies of the spleen the same changes are seen as in the follicles of the lymphatic glands. The Malpighian bodies become enlarged from the swelling of their cells and from the subsequent degenerative and necrotic process which begins at the centre. Single cells and small groups of cells may be seen degenerating in all parts of the pulp, but well-marked focal areas of degeneration are confined to the centres of the Malpighian bodies. Karyokinesis is rarely seen. The focal degeneration in the bone-marrow, like that of the lymphatic glands, is marked by areas of cells with fragmenting nuclei.

In the alimentary tract the lymphatic nodules and follicles are strikingly altered. In the swollen tonsils, areas of necrosis identical with those seen in the lymphatic glands are found. In the stomach the swollen follicles gave macroscopically a granular appearance to the gastric mucosa, and the microscope reveals in these swollen follicles most extensive areas of necrosis. But the most severe effects of the burn on the lymphatic apparatus are exhibited in the intestine. The follicles, both solitary and agminated, were in all the cases so swollen as to extend above the surface of the mucosa and laterally into the adjacent membrane (Plate XXXVI, Fig. 1). Yet on microscopic examination no cell division can be here made out in the lymphatic tissue. The degeneration of the lymphocytes in these follicles is like that of the more diffuse forms seen in the follicles of the lymphatic glands. The focalized areas of degeneration and necrosis are, however, of far greater size than those in the most altered lymphatic glands. In the lymphoid cells of the villi scattered single cells and groups of cells can be found undergoing fragmentary necrosis in the same manner as in the splenic pulp.

Flexner* has suggested that the focal pathological effects resulting from the action of certain poisons on the body may in some way be

* *The Journal of Experimental Medicine*, ii, 213 (1897).

related to the capillary circulation. I am persuaded that the degeneration in the lymphatic tissue in these cases of burn bears a similar relation to the blood circulation, though its exact nature in absence of experimental proof must be a matter of mere hypothesis. A possible explanation of this mechanism as regards the lymph follicle may, however, be offered.

But first let us glance at the blood supply of the lymphatic gland as demonstrated by Calvert.* He found that a small arteriole runs to the centre of each follicle and here breaks up into capillaries which radiate out from the centre and are collected into veins at the periphery of the follicle.

In the normal lymphatic gland it is probable that there is a steady flow of plasma from the centre of the follicle towards the periphery. But the normal plasma contains rich nutritive elements, and it is just at this central area of the follicle that reproductive cell activity is most active. From this centre of active cell formation lymphocytes are being continually pushed out towards the periphery, and thus it happens that in the area outside the germinal centre the lymphocytes are very closely packed. At the margins of the lymph follicles the normal lymph current finds its area of least resistance and works its way about, sweeping the lymphocytes at the periphery of the follicle into its current. This channel of least resistance is commonly known as the perifollicular lymph sinus.

The œdematous swelling of the follicles of the lymphatic glands in our cases of burn suggests strongly that the plasma escaped with much more than the normal rapidity from the capillaries radiating from the central terminal artery. The cell fragmentation in and about the germinal centre suggests that the lymphocytes first subjected to the influence of the escaping plasma were excited into a self-destructive activity. This violent necrosis of the lymphocytes it seems fair to ascribe to toxic substances in the plasma.†

* *Anatom. Anzeiger*, xiii, 174 (1897).

† We might wander too far into the realms of pure speculation were we to suggest a physiological explanation of this phenomenon. But if Uskow's ideas are correct as to the life history of the leucocytes, a physiological basis of the pathological process described above might be imagined to be

The lesions just described in the lymphatic tissue are essentially like those produced experimentally in animals by the injection of diphtheria toxin (Welch and Flexner*) and by abrin, ricin,† and other so-called toxalbumins. They are strikingly like those found in the lymphatic glands of children whose death has resulted from diphtheria. Indeed the changes seen in all the internal organs are very similar to those seen after fatal cases of those fevers, the main effects of which we ascribe to toxic substances circulating in the blood. Thus these lesions of the lymphatic tissue furnish most important additional evidence of a toxæmia after superficial burns. The character of the lesions produced by the poisons gives rise to the suspicion, at least, that these poisons are of the nature of the other highly complex poisons just mentioned. While swollen lymphatic glands have several times before been noted at autopsies after burns, the specific nature of the lesions has not, so far as we can find, before been mentioned. Swelling of the intestinal follicles was noted in dogs experimentally burned by Avdakoff.‡ Necrosis was noted in the bone marrow of animals experimentally burned by Foà.§

In our cases, if we may judge from the tissues examined, neither destruction of red blood corpuscles nor thrombosis played so prominent a part in producing death as did the poisons in the blood plasma.

The rapidity with which the lymphatic lesions developed in our cases is one of their most striking features.

It is always interesting to see ideas, which improved methods of

somewhat as follows: Bodily conditions marked by the presence in the blood of certain toxic substances are best met by a leucocytosis. The leucocytes are supplied in large part by the lymphatic glands. The presence of a certain amount of the toxic substances in the plasma bathing the lymphocytes causes an increased reproductive activity and thus indirectly an increase in the leucocytes of the blood. But in too large quantities the toxic substances, instead of causing increased reproductive activity, cause, as we find here, destructive activity at the centres of normal cell reproduction. It is possible also that the toxins cause the leucocytes to run through their life history with increased rapidity. The distorted nuclei of the polymorphonuclear leucocytes would rather favor this idea.

* *Bulletin of the Johns Hopkins Hospital*, 1891, No. 15, 1892, No. 20.

† Flexner, loc. cit.

‡ Dissertation. St. Petersburg, 1876.

§ *Rivista Sperimentale*, vii (1881), 135-143.

investigation bring into view, foreshadowed years before by the keen-sighted. Long,* a well-known English surgeon, as far back as 1840 stated that, clinically as well as from their complicated effects on the internal organs, burns closely resemble acute febrile diseases involving the skin.

In conclusion, I wish to express my thanks to Dr. Simon Flexner not only for the opportunity of studying these cases, but also for invaluable help in carrying on the work. I wish also to thank Dr. L. F. Barker for many useful suggestions, and the members of the surgical staff of the Johns Hopkins Hospital for many courtesies.

DESCRIPTION OF PLATES XXXVI AND XXXVII.

PLATE XXXVI.

**Fig. 1. Photomicrograph of section of small intestine showing swelling of lymph-follicles.**

**Fig. 2. Photomicrograph of microscopical section of centre of lymph-follicle, showing an early stage of focal necrosis.**

PLATE XXXVII.

**Centre of lymph-follicle, showing well advanced area of focal necrosis.**

* *London Med. Gazette*, N. S., i, 743.

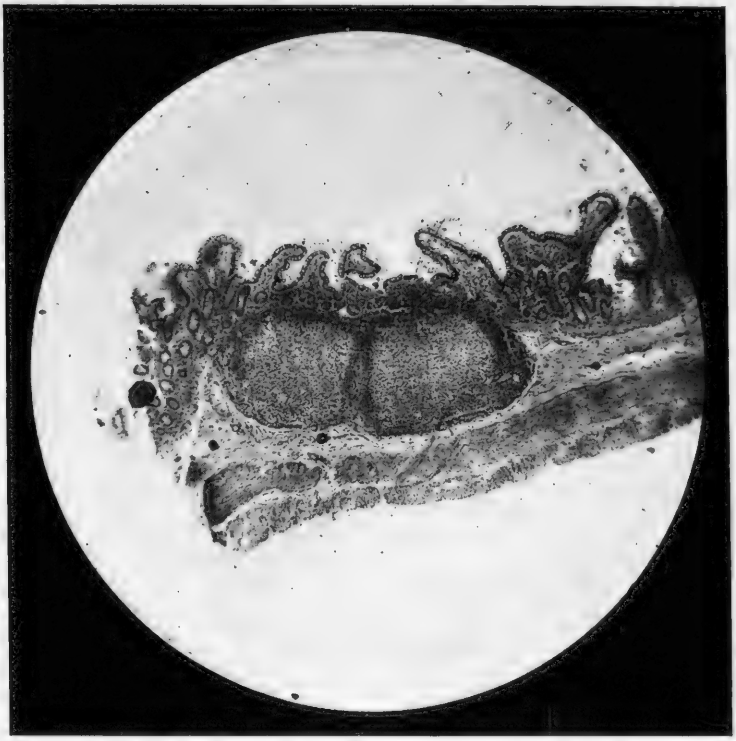
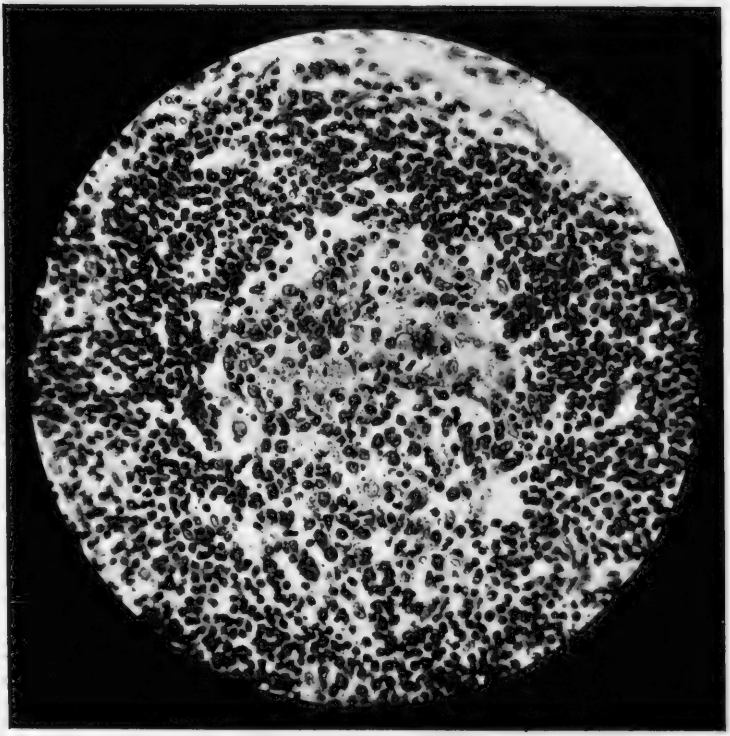
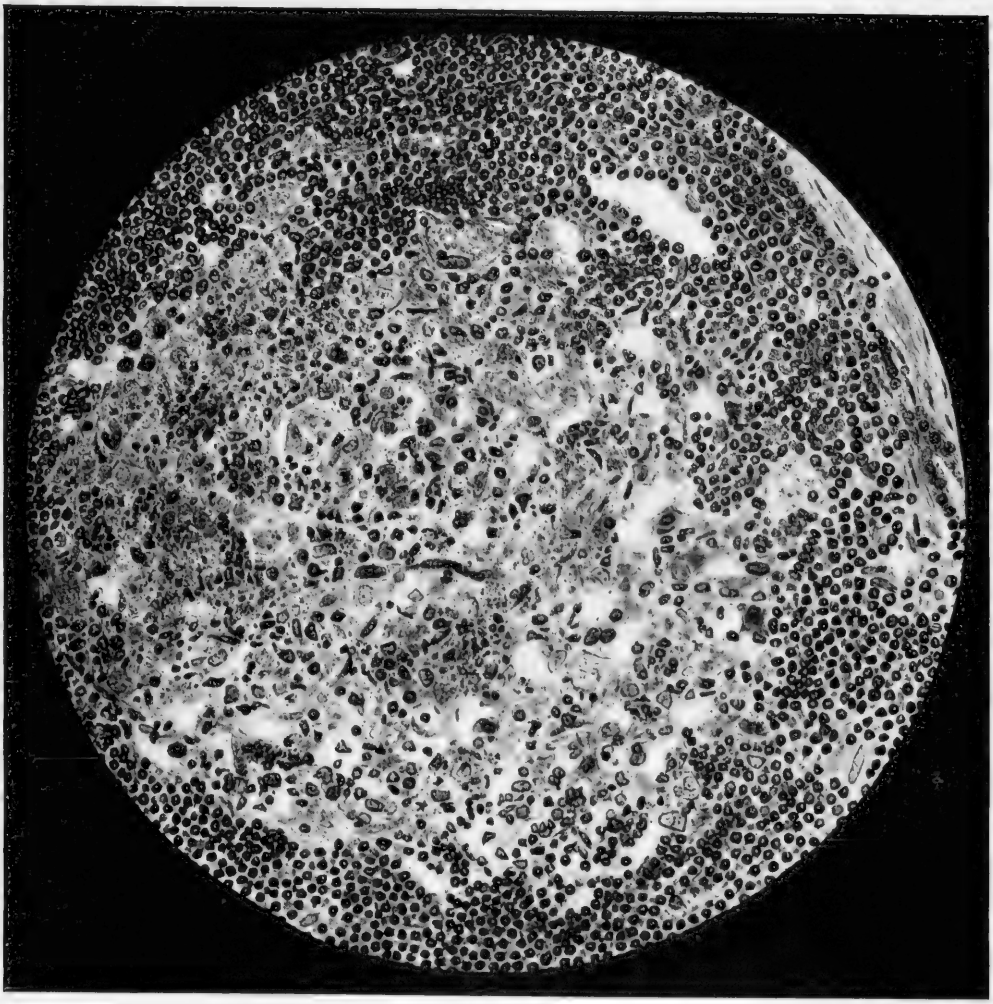


Fig. 1.





# ON THE APPEARANCE OF CERTAIN AMŒBOID BODIES IN THE BLOOD OF VACCINATED MONKEYS (RHOESUS) AND CHILDREN, AND IN THE BLOOD FROM CASES OF VARIOLA.

## An Experimental Study.

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Plates XXXVIII–XL.

Even prior to the introduction of Koch's improved methods, Cohn had described his micrococcus vaccinæ. Chauveau and Sanderson had demonstrated that the active ingredient contained in vaccine lymph could be removed by filtration.

The possibility of making use of solid culture media gave a new impulse to the search for the causative agent of vaccinia and variola, which has been assiduously continued since that time, as a rule, along the line of bacteriological research.

Notwithstanding the many claims of positive results, these have in the end proven groundless; unless one excepts the latest claim of Klein* (1) and Copeman (2) for the discovery, independently, in vaccine and variolous lymph of a short spore-bearing bacillus of peculiar staining properties and which refuses to grow on all of the usual media.

During the past ten years, and more especially during the last half-decade, attention has been turned toward the possibility of the animal nature of the parasite of vaccinia and variola.

Van der Loeff (3), in 1886, mentioned "numerous small bodies capable of independent movement" which he discovered while examining the clear fresh lymph of a calf, as a hanging drop, in a warm chamber. He gives no other description of these bodies which he assigned to the class Rhizopoda.

If the lymph had been taken with antiseptic precautions these bodies were still to be found after months had passed; but if bacterial contamination of the lymph had occurred they were not to be seen after the lapse of one or two weeks. Impure lymph in which these little bodies could

* References to literature are at the end of this article.

not be found was inactive, whereas the inoculation of pure lymph containing them was always followed by success, even at the end of several months. The following year the same observer (4) reported the presence of similar amœboid bodies, only more numerous, in lymph taken from two cases of small-pox at the commencement of the stage of pustulation.

Shortly after van der Loeff had published his observations, L. Pfeiffer (5) called attention to certain unicellular, round or oval bodies constantly present in the lymph of vaccinia and variola, which he believed to be parasites and assigned to the sporozoa (Leuckart), order Monocystis.

These parasites develop in the cells of the Malpighian layer of the skin, rapidly forming into cyst-like bodies. Propagation takes place by the formation of spores within the cyst wall. The mature spore presents the appearance of a minute disc with vacuolation, out of which there appears to be developed an amœba-like embryo having independent movement. In 1888 Pfeiffer (6) found in human lymph numerous pale hyaline discs, without a nucleus, which exhibited rapid amœboid movements. Rieck (7) confirmed Pfeiffer's finding in human, calf's and variolous lymph. More recently (1895) Ogata (8) has made careful microscopic examinations of stained cover-slip preparations of fresh child's lymph. As a result of his studies he finds in humanized, bovine and variolous lymph certain protozoa which belong to the class Gregarinida, family Polycystidæ, genus Clepsidrina, and is of the opinion that these are probably the causative agent of vaccinia and variola.

The first attempt to cultivate the supposed parasite of vaccine lymph was made by Guarnieri (9) in 1892. He first studied the changes which occur in the epithelial cells of the skin during the prepustular stage of small-pox. He always found certain small bodies half the size of the nucleus, or much smaller, which occupied the protoplasm of the affected epithelial cells and stained uniformly and regularly. The little bodies were of various shapes, most often roundish, with irregular margins.

Guarnieri next inoculated the cornea of guinea-pigs and rabbits with vaccine lymph and obtained characteristic lesions, due to the development of a parasite within the deep epithelial cells of the cornea. At various periods of development—generally at the end of 48 hours—scrapings from the cornea were made and examined while suspended in a hanging drop of aqueous humor. Thus prepared, Guarnieri always found within the epithelial cells of the affected area little shining, slowly amœboid bodies, which occupied the protoplasm of the cell but did not obtrude upon the nucleus. Stained sections of the cornea, made through

the site of inoculation, examined under 500 diameters, showed that while the superficial cells were not involved, almost every deep epithelial cell contained, besides its nucleus, a small deeply stained body which lay in the protoplasm and was surrounded by a narrow clear zone. Each cell, as a rule, contained one of these bodies, though two or more might be present in the same cell. Examined under one thousand diameters these bodies were seen to consist of a round or oval nucleus, surrounded by a layer of protoplasm which assumed various shapes. Guarnieri considers these bodies to be parasites and assigns them to the protozoa. From the fact that the parasite occupies the protoplasm of the cell, which it destroys as it develops, Guarnieri designates it Cytoryctes variolæ seu vaccinæ.

Ferroni and Massari (10), in 1893, reported that after inoculation of the cornea of guinea-pigs and rabbits with croton oil and India ink, small bodies, like those described by Guarnieri, could be found in the epithelial cells, but in smaller numbers. They believe that the so-called parasites of Guarnieri are derived from the nucleus of the cells or from emigration of leucocytes.

In 1894 L. Pfeiffer (11) confirmed Guarnieri's experiments on the cornea of animals, and announced the discovery of the parasite also in the blood of small-pox patients, and in that of vaccinated children and calves during the stage of fever. He described these parasites as amœboid cells, one-half the size of a red blood cell in the calf and one-fourth that of a red blood cell in the child. A nucleus is present which can be stained; sometimes the parasite contains from 2 to 4 nuclei. They are provided with pseudopodia and often with flagella that can be stained with Loeffler's flagella stain. These bodies are not intracellular, but swim free in the blood. The movements of the parasites are different from those of mononuclear and polynuclear leucocytes and resemble closely those of amœbæ. According to Pfeiffer there are two stages in the development of this parasite. The first consists of an infection of the protoplasm of the epithelial cells by those bodies which were first cultivated in the corneal epithelium by Guarnieri. The amœbæ found during the stage of fever in the blood of vaccinated children and in the small-pox blood belong also to this stage. Out of the young form occupying the epithelial cells develops the second stage, which presents itself as a kind of cyst—*Monocystis epithelialis* of Pfeiffer—whose contents divide into a number of spores. The further development of the spore into the young form has not as yet been followed.

The following year (1895) Von Sicherer (12), Clarke (13) and E. Pfeiffer (14) published confirmatory observations of Guarnieri's experi-

ments on the cornea. Von Sicherer pronounces the process to be a local infection of the corneal cells by a parasite, which, while destroying the protoplasm of the cell, leaves the nucleus undisturbed. E. Pfeiffer failed to find these bodies in the corneal epithelium of rabbits inoculated with chemical irritants, such as osmic acid, glycerine, croton oil and lunar caustic; but they were uniformly present in the cornea of rabbits, goats and calves inoculated with vaccine lymph. Pfeiffer also found in the blood of vaccinated calves amœboid bodies which continued their movements for several hours. His description is meagre, but his figure (No. 6) shows homogeneous amœboid bodies of various shapes, generally with one nucleus. These bodies approach in size a red blood cell.

The foregoing embraces a brief review of the results obtained from a microscopical study of the local lesions of the skin in vaccinia and variola, and of the lesions of the cornea produced by inoculation with vaccine lymph. While not conclusive, the weight of testimony points strongly to the appearance within the corneal epithelium, following local vaccination, of certain definite bodies, possessing independent amœboid movements, and which do not appear to be called forth by various chemical irritants.

As regards the claim of L. Pfeiffer that small, free-swimming, nucleated amœboid cells appear in the blood of variola and vaccinia during the stage of fever, which bodies he considers as the much sought parasite of these diseases, nothing confirmatory has so far been published.

At the suggestion of Surgeon-General Sternberg I took up this line of investigation about one year ago, directing attention at first to the microscopical examination of the blood of vaccinated monkeys and calves, and later to that of children and small-pox patients.

The work was begun in April and continued with slight interruptions until September, 1896. Except in the examination of the blood of small-pox patients, I was assisted by Dr. W. W. Gray, of the Army Medical Museum, and Dr. James Carroll, Assistant in the Laboratory of the Army Medical School, to the former of whom my thanks are also due for microphotographs and drawings, and to the latter for daily valuable assistance in the blood examinations covering a period of nearly five months.

The investigation was entirely confined to the study of fresh preparations of blood which were examined with the greatest care, one at least of the observers making use of a mechanical stage for this purpose. During the earlier experiments the blood was examined only at the height of vaccination (seventh and eighth day). In the later experiments the period of examination was extended to cover the time from the day of vaccination to the subsidence of this process, and in several of the experiments the blood was examined during a number of days preceding and following vaccination.

The results obtained are believed to be of sufficient interest to warrant their report.

In all the vaccinations, except one, glycerinated calf's vaccine was used. In the excepted case fresh monkey's vaccine was inoculated.

The examination of the four animals—three monkeys, one calf—first experimented upon was entirely negative. The succeeding experiments gave more encouragement. The following are brief details of some of these:

*Experiment V.*—Monkey. Vaccinated May 5, 1896. Successful. Daily examinations of the blood were made, beginning on the 4th day after vaccination. These are negative until the sixth day, when a body is found which attracts attention. It is in diameter about one-third that of a red blood cell; its protoplasm consists of very fine granules, these being smaller than those of an ordinary neutrophilic cell. From the periphery of this small body, clear, short, blunt bits of protoplasm are being continually projected and withdrawn. The granular portion of the protoplasm does not at any time flow into these clear pseudopodia, although the body changes its general shape, being sometimes round or oval, or even triangular. At times a round or oval white spot resembling a nucleus appears in its granular protoplasm. It is kept under observation about six hours (cold stage), during which time its amœboid movements continue active, but the body does not change its position. Other bodies of like granulation and size, some quiet, some amœboid, are observed. One of these bodies seen on the 8th day and left in the field of the microscope during the night shows at the end of 23 hours two distinct, long curved processes resembling flagella. Its amœboid movements have ceased and its granular protoplasm appears darker and more condensed. On several occasions a neutrophilic leucocyte is seen to approach one of these bodies, envelop it completely, and then, after

an interval of a few minutes, release the body and pass on. These small granular bodies are found by each of three observers on all slides examined on the 7th, 8th, 9th, 10th and 11th day after vaccination (Photomicrographs 1–6, Plate XXXVIII). Their number is not large, but as many as five can be found on one slide.

They cannot be found on the 12th, 13th and 14th day, although patient search is made.

*Experiment VIII.*—Calf. Vaccinated May 13, 1896. Successful. Examination of blood from first to eighth day. On the third day after vaccination there are seen a few finely granular bodies, in diameter one-fourth to one-half that of a red cell, which are either quiet or slowly change their general shape without projecting any clear protoplasm. Two similar bodies, however, are actively amœboid. These bodies continue to appear until the seventh day, when they can no longer be found. These small amœboid bodies, both as to appearance and character of movement, resemble closely those seen in Experiment V.

*Experiment XI.*—Monkey. Vaccination June 6, 1896. Successful. This animal's blood had been examined daily by two observers from May 22nd to June 5th with negative results. There are seen, however, prior to vaccination, from time to time, small, roundish bodies having a slightly greenish refraction and no distinctly granular protoplasm. These are in diameter about one-third that of a red cell and possess amœboid movements. Small, short processes are projected which quickly assume the same greenish color as that of the body proper. They are not to be confounded with the granular bodies seen in vaccinia. After vaccination daily examinations are negative till June 12th (sixth day). On this day the granular amœboid bodies appear in the blood in moderate numbers. They are in all respects similar to those described in Experiment V, except that some of them are smaller, having scarcely a diameter of one-sixth that of a red cell, and some have a slightly coarser and darker granulation. Distinct, clear, blunt processes are projected by these bodies into which, as a rule, the granular protoplasm does not flow, though exceptionally this is seen to take place. Several of these granular bodies are still amœboid at the expiration of thirty hours. On the 12th day a body unlike any heretofore seen in monkey's blood is found. Its diameter measures one-half that of a red cell, and the body consists of a number of dark granules situated in the midst of a clear protoplasm. This body is rapidly amœboid, its clear periphery continually changing form and sometimes assuming rosette shapes (Plate XL, Figs. 1–9). A few granular bodies are present on the 14th day. Examination on the 15th, 16th and 17th days negative.

Thus of eleven animals—9 monkeys, 2 calves—the blood of seven—6 monkeys and 1 calf—contained during the stage of fever attending a successful vaccination certain extra-cellular, small, granular amœboid bodies which did not correspond to any of the normal elements of human blood. A fact which appeared of additional interest was the discovery of quite similar amœboid bodies in my own blood five days after an accidental vaccination on the finger with fresh monkey's lymph. These bodies were present till the 10th day, but could not be found thereafter.

The time of appearance of the bodies in the blood of the vaccinated monkeys was as follows: 5th day, 2; 6th day, 4. There was observed, however, in the blood of one of the monkeys (Experiment 10), 20 hours after vaccination, an amœboid granular body similar to those which had been seen to appear toward the end of the first week after vaccination. No other bodies were found thereafter in this particular animal's blood until the 6th day. The bodies disappeared as follows: 12th day, 3; 13th day, 1; 14th day, 2. In the one positive experiment with the calf they appeared on the 3rd day and could not be found after the 7th day.

As these rather interesting bodies had been found in the blood of the seven animals last experimented upon, it was considered advisable to continue the investigation with vaccinated children. In this second series of experiments the blood of five children was examined —3 white, 2 black. The following are brief protocols of some of these:

*Experiment XII.*—Child, 4 yrs., white. Vaccination June 23, 1896. Successful. Examination of the blood on the day preceding vaccination is negative. Examination of blood taken thirty minutes after vaccination shows one small, faintly granular body, in diameter one-third that of a red cell, which sends off clear, blunt processes after the manner of the bodies already described in the vaccinated monkey's blood. From this time until the 7th day examinations are negative. On this day there are observed a few pale amœboid bodies, with a diameter one-half that of a red blood cell, containing a few fine, dark dancing granules and which are slowly amœboid. Similar bodies are observed on the 9th day. Examination on 10th, 11th and 12th days is negative.

*Experiment XIV.*—Child, 2 yrs., negro. Vaccination July 8, 1896. Successful. Blood subjected to daily examinations for one week preceding vaccination, with the result that four days prior to vaccination there is found a finely granular, actively amœboid body, in diameter one-third that of a red blood cell, which sends off clear processes into which the granular protoplasm is not seen to flow. Twenty-four hours after vaccination a similar body is found. The results are then negative until the 7th day, when the small granular amœboid bodies, as heretofore seen in monkey's blood, can be found in all fresh preparations (Photomicrographs 7–12, Plate XXXIX).

Sometimes as many as nine of these bodies are found on one slide. There is also seen on the 9th day a pale, slightly refractive body, with a diameter one-third that of a red cell, which contains one coarse, dark granule centrally situated, while its clear periphery undergoes continual changes of shape (Plate XL, Figs. 10–16). Examination of the blood on the 14th, 15th and 16th day is negative. In this child's blood prior to and during vaccination the small amœboid body with slightly greenish refraction, which has been seen in the normal blood of the monkey, was found on several occasions.

Thus of five children whose blood was carefully studied during the period of vaccination, three (2 black, 1 white) gave a positive and two (both white) a negative result, as far as the finding of the small granular, amœboid bodies was concerned. The case (Experiment XII) in which a granular body was seen in the blood taken thirty minutes after vaccination, but did not appear thereafter, is classed as negative. The bodies appeared in two cases on the 6th and in one on the 7th day; they disappeared from the blood in two cases on the 14th and in one case on the 17th day.

When the latter experiments were about completed the occurrence of an epidemic of small-pox on the island of Key West, Fla.,* afforded the opportunity for the microscopic study of the blood of this disease. Unfortunately for these studies, upon my arrival the epidemic had about spent itself, so that only three cases could be examined, with the following result:

* Through the courtesy of Dr. J. Y. Porter, State Health Officer, and Dr. Jos. H. White, Passed Asst. Surgeon, U. S. M. H. Service, officer in charge of the small-pox hospital, Key West, Fla., every facility was afforded the writer in conducting this investigation.

*Observation XVII.*—White boy, 11 yrs. Discrete small-pox. 7th day of eruption. The same granular bodies, both as to size and amœboid movements, as seen in the blood of vaccinated monkeys and children are found. In addition there are observed on each slide pale, amœboid bodies, few in number, which contain a few fine or sometimes coarse refractive pigment-like granules. The size of these pale bodies is about one-half that of an ordinary red blood cell. They are generally actively amœboid, continually changing their outline and sometimes assuming rosette-shaped forms. These amœboid bodies are present on the 8th day. One of these is kept under observation for several hours. Its granules, few in number, remain centrally situated, while its periphery is rapidly changing, clear processes being quickly projected and withdrawn. It is left in the field of the microscope during the night and fifteen hours later presents the appearance of a quiet round body, in diameter one-half that of a red cell, with a pale, refractive peripheral zone and a granular centre, the latter made up of a number of quite dark refractive granules (Plate XL, Figs. 17–25).

Examination of the blood on the 9th day is negative.

*Observation XVIII.*—Negro, 11 yrs. Discrete small-pox. 4th day of eruption. The granular amœboid bodies and the pale amœboid bodies, such as have been described in the preceding observation, are found daily from the 4th until the 7th day of eruption. No examination made on the 8th day. On 9th and 10th days the examination is negative.

*Observation XIX.*—Negro, 70 yrs. Confluent small-pox. 8th day of eruption. No granular amœboid bodies are seen in this blood. Pale, amœba-like bodies are observed. These vary in diameter from one-third to one-half that of a red cell. Kept under observation for a considerable time, they show frequent changes of shape and contain a few fine, dark dancing granules. One body particularly arrests attention. It contains within its pale protoplasm a few very fine pigment-like granules—eight at most can be counted—which are, as a rule, gathered towards the centre, though they may be at times scattered. This body is very active in its movements, remarkably large blunt pseudopodia, as many as four at one time, being rapidly sent forth in various directions. Two hours later it has assumed the form of an oval amœba one-half the size of a red cell and changes its general shape very slowly. The dark granules are no longer gathered towards the centre, but are distributed throughout the body. This patient died the same day, so that further examination could not be made.

Thus of three cases of variola, the small granular amœboid bodies were present in the blood of two and absent in that of one (blood examined on the 8th day of eruption only). Paler amœboid bodies were found in the blood of three, their appearance in one case being as early as the 4th day of eruption. In two cases they were found on the 7th and 8th day of eruption, respectively. In two cases they were not found on the 9th day.

In view of the results obtained from a study of the blood of variola, it was considered advisable to study the blood of variolated monkeys. Hence soon after my return the following experiment was made:

*Experiment XX.*—Monkey. Variolated August 8, 1896, with fresh lymph taken from a discrete case of small-pox, 7th day of eruption. Successful. This animal's blood had not been examined prior to inoculation. First day's examination is negative. On the 3rd day after vaccination small finely granular amœboid bodies are observed. These are seen on the 4th, 5th and 6th days. There are also found on the 5th and 6th days a few pale amœboid bodies, in diameter one-half that of a red cell. These exhibit active movements and contain a few dark pigment-like granules (Plate XL, Figs. 26–30).

They resemble the pale amœboid bodies seen in the blood of variola.

Examination on the 7th, 8th and 9th days is negative.

In view of Pfeiffer's statement that small amœboid cells are to be seen during the stage of fever in the blood of vaccinated children and calves, the discovery in the blood of vaccinated monkeys, also, during the stage of fever, of granular amœboid bodies much smaller relatively than any of the white elements heretofore described in normal human blood, naturally aroused much interest as to their true character. Their disappearance, too, about the end of the second week did not lessen the important and intimate relation which these bodies appeared to bear to the local inflammation. Their minute size, their active amœboid movements, the persistency of the latter, together with the occasional phenomenon of phagocytosis, all seemed to point to the parasitic nature of these bodies, as claimed by Pfeiffer.

The discovery, however, of a similar body in the blood of a monkey within twenty-four hours after vaccination, and in the blood of a child

thirty minutes after vaccination, necessarily raised a doubt as to their causative importance. It seemed possible, after all, that such bodies might be occasionally found in normal blood and that during vaccination they might appear in larger numbers in the general circulation. This led to a thorough search for these diminutive bodies in the normal blood of monkeys and children. As a result of this search there was found in the blood of one monkey and of one negro child, prior to vaccination, a body having the same appearance and size as those seen in the blood of vaccinated animals. The failure to observe this small granular body heretofore in normal blood must be attributed to its extreme scarcity and relatively small size. The tendency, too, to overlook such small bits of protoplasm would be natural, since on casual inspection they might readily be mistaken for parts of a fragmented leucocyte.

The origin of these small granular bodies, rarely seen in normal blood, as well as their possible identity with those bodies observed by Pfeiffer and myself in the blood of vaccinated animals and children and in that of small-pox, must be left to future observation to determine. Their appearance in the general circulation in vaccinia about the end of the first week, in such numbers that a few may be found in every well-made preparation of blood, and their prompt disappearance about the end of the second week, is indicative of their importance, whatever may be their real significance.

The pale amœboid bodies which were observed in the blood of three cases of small-pox and of one variolated monkey will, it is hoped, attract the attention of other investigators. Bodies such as these have not been found, during this investigation, in the normal blood of animals or children.

### CONCLUSIONS.

1. The claim of L. Pfeiffer that small granular amœboid bodies are present in the blood of vaccinated children and calves, and in the blood from cases of variola, during the stage of fever is confirmed. A nucleus has not been positively made out in any of these bodies.

2. Granular amœboid bodies, having a diameter about one-third that of a red blood cell, appear, also, in the blood of the monkey

during the active stage of vaccinia and disappear with the decline of the local inflammation.

3. A body of like appearance, granulation and size may occasionally be found in the normal blood of monkeys and children.

4. Pale amœboid bodies, containing a few dark pigment-like granules, are present in the blood from cases of variola and in the blood of the variolated monkey. Bodies of like appearance may occasionally be found in the blood of vaccinated children and monkeys.

DESCRIPTION OF PLATES XXXVIII–XL.

The description accompanies the plates.

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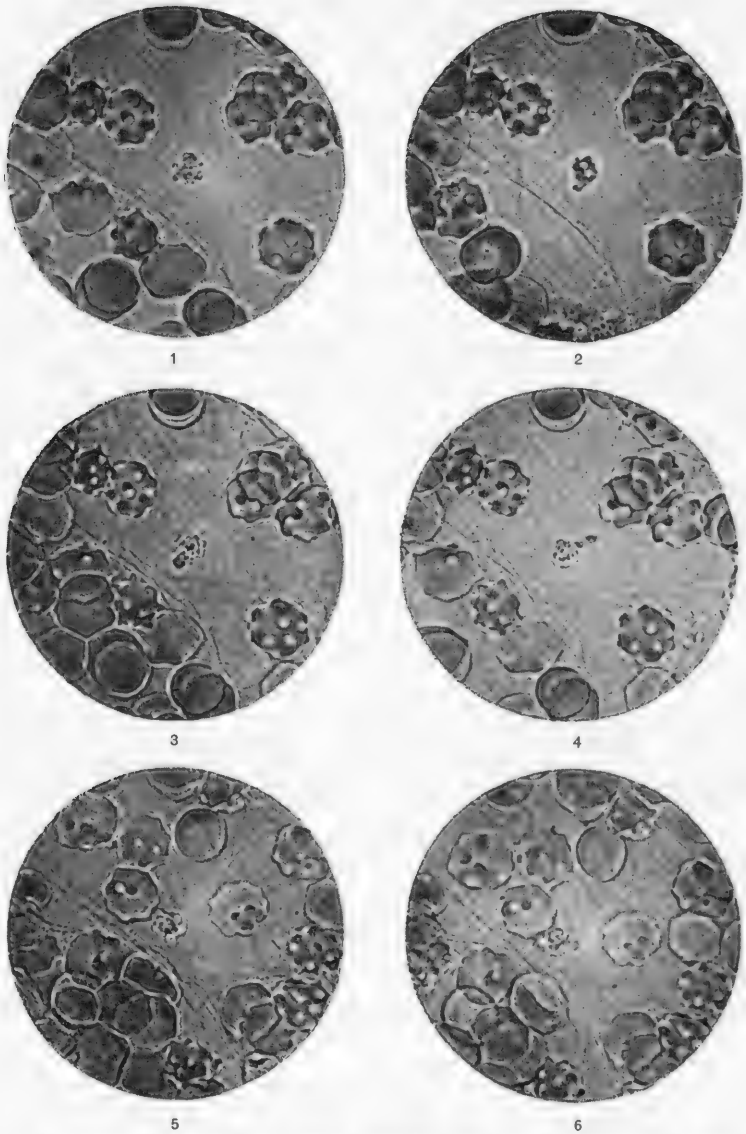
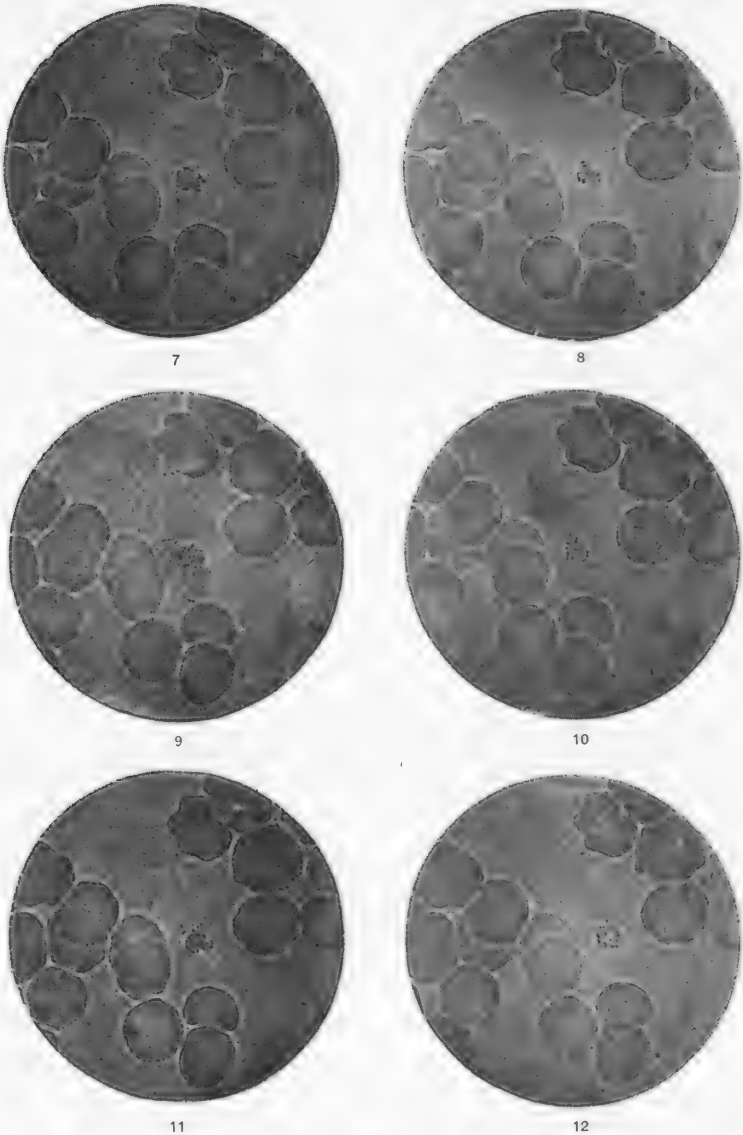



A granular amœboid body, blood of monkey, 8th day of vaccination. Photomicrographs taken at intervals of about thirty seconds. × 1000.




A granular amœboid body, blood of negro child, 8th day of vaccination. Photomicrographs taken at intervals of about thirty seconds. × 1000.

# ON CERTAIN IMPROVEMENTS IN HISTOLOGICAL TECHNIQUE.

I. A DIFFERENTIAL STAIN FOR AMŒBÆ COLI.
II. PHOSPHOTUNGSTIC-ACID-HÆMATOXYLIN STAIN FOR CERTAIN TISSUE ELEMENTS.
III. A METHOD OF FIXATION FOR NEUROGLIA FIBRES.

By F. B. Mallory.
(*From the Scars Pathological Laboratory of Harvard University.*)

Plate XLI.

## A DIFFERENTIAL STAIN FOR AMŒBÆ COLI.

The recognition of amœbæ coli has depended in part on their rather characteristic morphology, but chiefly on their movements as shown by the extrusion of pseudopodia. Examination of the organism in the fresh state, therefore, has been indispensable, and a positive diagnosis has rested on the exhibition of the characteristic amœboid movements.

In hardened tissues amœbæ coli are usually recognized with difficulty. Their nuclei do not stain with the ordinary basic stains, such as methylene blue and alum hæmatoxylin. The following method not only stains the nuclei of the organisms, but brings them out in a color entirely different from that of the nuclei of other cells. It is believed that this differential stain renders possible the recognition of amœbæ coli in tissues in the same way and with the same certainty that the tubercle bacillus is recognized by its characteristic stain.

### DIRECTIONS FOR STAINING.

1. Fix tissues in alcohol.
2. Stain sections (celloidin or paraffin) in a saturated aqueous solution of thionin for 5–20 minutes.
3. Wash in water.

4. Differentiate in a 2 per cent aqueous solution of oxalic acid for ½ to 1 minute.

5. Wash in water.

6. Dehydrate in 95 per cent alcohol.

7. Clear in oleum origani cretici or in oil of bergamot.

8. Wash with xylol.

9. Xylol balsam.

For paraffin sections use absolute alcohol and clear directly in xylol.

The nuclei of the amœbæ and the granules of mastzellen are stained brownish red (Plate XLI). The nuclei of mastzellen and of all other cells are stained blue. If the sections are washed out too long in alcohol the nuclei of the amœbæ assume a light and more brownish tint, and the protoplasm is colorless. By decolorizing the sections to a less extent the nuclei of the amœbæ are of a deeper red, and the protoplasm is often tinted bluish or purplish, so that it can be more easily seen. The nuclei of the amœbæ stand out in such marked contrast to those of other cells that they are readily recognizable with comparatively low powers.

In a case of amœbic abscess of the liver it was found easily possible to stain small fragments of the softened material which had been hardened in alcohol (Plate XLI, Fig. 4). The stained bits were teased into still smaller fragments after they were in balsam. In this way a positive diagnosis of the presence of amœbæ coli was quickly and easily made.

With hardened dysenteric discharges the results obtained with the same method of staining were only fairly satisfactory, mainly because many substances present in the fæces precipitate thionin in the form of reddish crystals. Attempts to stain cover-slip preparations proved wholly negative.

It is possible to obtain a similar differential stain for amœbæ coli by using Unna's method for staining differentially the protoplasmic granules of mastzellen. His method is as follows:

1. Harden tissues in absolute alcohol.

2. Stain sections in Unna's polychrome methylene blue solution (Gruebler) ¼ hour to over night.

3. Decolorize and differentiate in a small dish of water to which are added a few drops of glycerine-ether mixture (Gruebler).
4. Wash thoroughly in water.
5. Alcohol.
6. Oil of bergamot.
7. Balsam.

It is possible that this second method would give better results with intestinal discharges.

### PHOSPHOTUNGSTIC-ACID HÆMATOXYLIN.

The following solution of hæmatoxylin is a more or less perfect differential stain for certain tissue elements and possesses the peculiar property of being polychromic:

Hæmatoxylin .....0.1 gramme.
Phosphotungstic acid (Merck) 1 per cent aqueous solution.....100 cc.

Dissolve the hæmatoxylin in a little hot water and add when cool to the dilute acid solution. The color, at first greenish, turns in a few minutes to a reddish brown of little intensity. The solution is ready for use at once, and will keep for several months if not exposed to too much light.

### DIRECTIONS FOR STAINING.

1. Stain sections 2–24 hours.
2. Wash in water.
3. Dehydrate in alcohol.
4. Clear in oleum origani cretici.
5. Xylol balsam.

Although the time required for staining is long, a stronger solution has not been found advisable. Prolonged washing in alcohol will remove most or all of the pink color. Celloidin remains unstained.

After any fixative, nuclei by this method are stained blue, while connective tissue fibres and the intercellular substances of bone, cartilage and the cornea are stained a light to a deep pink. If the staining is prolonged the cell protoplasm is usually colored a light blue.

Besides the nuclei, certain other tissue elements and products stain blue. Fibrin, the contractile elements of striated muscle fibres and elastic fibres stain sharply after fixation in alcohol, less well after Zenker's fluid. Bile capillaries often stain sharply after fixation in Zenker's fluid. Possibly better results would be obtained from the method used for neuroglia fibres. Smooth muscle fibres sometimes stain after Zenker's fluid, but the results are not constant. Red blood globules generally take a dark greenish tint.

For the central nervous system the stain will be found useful after fixation by the method already published, and given here again slightly changed, for mordanting neuroglia fibres so that they will stain by the fibrin method. The mordanting with the chrome salt must be as long as when a stain of the myelin sheath is desired, otherwise the myelin will stain to some extent with this solution.

The method is as follows:

METHOD OF FIXATION FOR NEUROGLIA FIBRES.

1. Fix thin pieces of tissue, not over $\frac{1}{2}$ cm. thick, in a 4 per cent aqueous solution of formaldehyde (10 per cent solution of formaline) for 4 days or longer.

2. Saturated aqueous solution of picric acid, 4–8 days. Steps 1 and 2 may be combined by adding the formaldehyde to the picric acid in proper proportion.

3. 5 per cent aqueous solution of bichromate of ammonium, 4–6 days in the incubator at 37° C., or for four weeks at room temperature. Change the solution on the second day.

4. Transfer to alcohol without washing.

5. Imbed in celloidin.

Sections may be stained by Weigert's fibrin method (decolorizing, however, with equal parts of aniline oil and xylol), or with the phosphotungstic-acid-hæmatoxylin solution. With the latter solution the neuroglia fibres which have been properly fixed and mordanted stain a deep blue. The nuclei stain blue, the protoplasm of ganglion cells a pale bluish or purplish gray, elastic fibres and fibrin blue, axis cylinders a dull pink, connective tissue a deep pink, myelin sheaths yellow (from chrome salt).

The stain is useful because all the various tissue elements are stained. For the study of individual neuroglia fibres and their relation to the cell protoplasm the stain is not so good as the modified fibrin method.

A previous light stain with Van Gieson's picro-acid-fuchsin mixture is often useful, because it makes the red of the axis-cylinder more pronounced and the dendritic processes stand out in sharper contrast to the neuroglia fibres.

Very good stains of some of the neuroglia fibres can often be obtained with this solution after simple fixation in formaldehyde, alcohol or Zenker's fluid.

It is important to bear in mind that good results with stains for neuroglia fibres can be obtained only with tissue that is perfectly fresh at the time it is placed in the fixing solution. For example, nearly perfect results can be expected from thin pieces of tissue placed in formaldehyde within one hour after death. Up to twelve hours *post mortem* fair results may be expected. After twenty-four hours the results are practically nil.

The first (surface) sections will always give the best results, because in them the neuroglia fibres are soonest fixed, and the peculiar chemical property which quickly disappears *post mortem* and on which the stains depend is preserved.

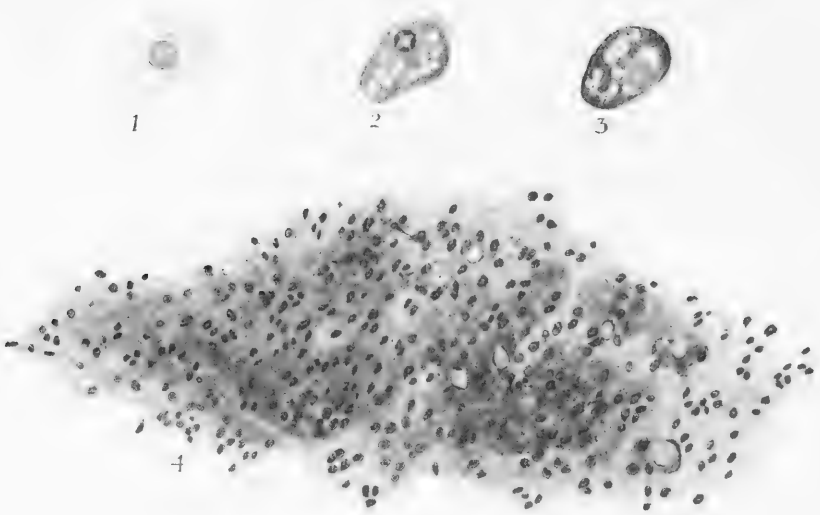
The above stain succeeds with human neuroglia fibres only.

DESCRIPTION OF PLATE XLI.

Figs. 1, 2 and 3 show amœbæ in dysenteric discharges, Fig. 1 after hardening in alcohol, Figs. 2 and 3 after fixation in corrosive sublimate.

Fig. 4 is a teased fragment from the purulent material obtained from an amœbic abscess. Alcohol hardening.

Fig. 5 is from the muscular coat of the intestine and shows three amœbæ and three mastzellen.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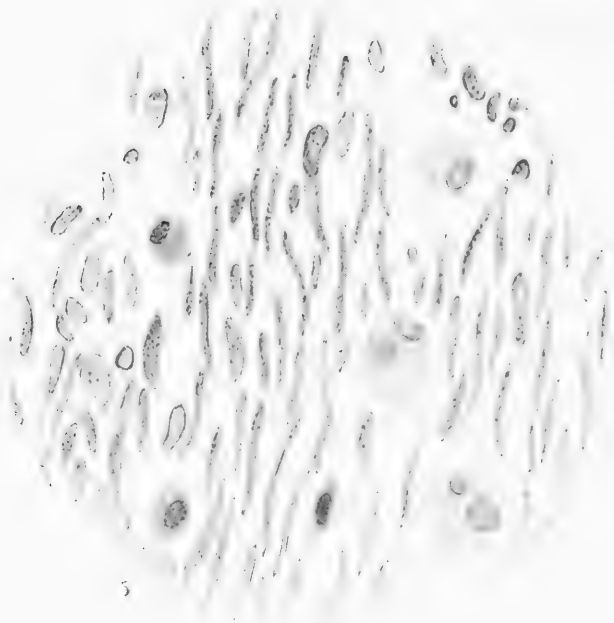
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# FURTHER STUDIES UPON THE PATHOGENIC SPIRILLA OF THE SCHUYLKILL RIVER AT PHILADELPHIA.

By A. C. ABBOTT, M. D., and D. H. BERGEY, M. D.
(*From the Laboratory of Hygiene, University of Pennsylvania.*)

In a former paper* by one of us (A. C. A.) attention was called to the fact that in the water of at least one American river, viz. the Schuylkill, at Philadelphia, pathogenic spirilla had been detected that possess the majority of the characteristics common to that group of spirilla whose most important member is the spirillum of Asiatic cholera. In the course of the investigation several questions arose, as to the frequency with which this variety was to be detected, its distribution in the river, and the possibility of its detection in water from other sources. Since the publication of these researches a part of the time at our disposal has been utilized in endeavoring to answer these questions. The results are embodied in this communication.

*First:* With regard to the question of constancy of occurrence. Is the presence of this organism in the river subject to seasonal variations?

In the studies of Dunbar upon the spirilla of the Elbe at Hamburg it was found that with the advent of autumn and winter, *i. e.* the onset of cold weather, the spirilla gradually diminished in frequency, and finally disappeared entirely from the water itself, though from time to time they were demonstrable in the mud from the bottom of the river.

Our studies, which have been continuous since June last, demonstrate the occurrence of spirilla in varying numbers in the water during all four seasons of the year. They were most numerous during the months of June, July, August and September, after which they gradually became fewer, and during January and February of this year almost, though not quite, disappeared; becoming again more conspicuous with the beginning of March.

* *The Journal of Experimental Medicine*, i (1896), 419.

Whether the diminution observed during the autumn and winter months can be attributed to the low temperature of the water alone, we cannot say, but we incline to the opinion that this, together with the frequent flushing of the river and its tributaries, particularly the sewers, by the heavy rains of autumn, has much to do with it.

*Second:* As to the distribution of the spirilla in the river. We have isolated them from samples of water collected at points situated a short distance apart, from practically all that part of the river that is within the city. They have been found at both high and low tides. We have also obtained them from a number of main and branch sewers that empty into the river on either side. They have also been isolated from the water drawn from the laboratory faucets, so that their presence in the river above the Fairmount dam, that is, above the points of greatest sewage pollution, is certain. Concerning this latter point, however, it must be remembered that the water of the Schuylkill river, as pumped into the city reservoirs and mains, to be used for domestic purposes, is already charged, to a certain extent, with sewage from towns located farther up the stream.

*Third:* Concerning the presence of this group of spirilla in other American streams: the time at our disposal has permitted the investigation of but one other source of supply, viz. the Delaware river. Here we encountered the spirilla as frequently as we did in the Schuylkill and under essentially similar conditions. The spirilla isolated from the Delaware river and the sewage emptying into it did not differ in nature from those found in the Schuylkill and its tributary sewers.

An interesting question that arises in connection with these investigations relates to the origin of these bacterial forms. We have, as stated, encountered them both in the waters of the two rivers and in the effluents from sewers emptying into them. Do they occur in the rivers as a consequence of sewage pollution, or are they in the sewage as a contamination by the river water that in some cases backs into the sewers during high tides? We are inclined to the opinion that they are brought into the rivers with the sewage, but from what source they gain access to the sewage we cannot say. It would be interesting

to know if in household slops, or in intestinal evacuations, either normal or pathological, such forms are to be commonly detected. We have always found them to be more numerous in sewage than in water from the middle of the rivers, but this might readily be referable to the more favorable opportunities that sewage, rich in nitrogenous matters, offers for their multiplication.

In all we have isolated one hundred and ten pure cultures of spirilla from the several sources mentioned. After subjecting them to the routine tests it was found that few or none of them were identical in every particular. At the same time they approached one another in so many features and to such varying degrees that we could not avoid the opinion that they were but modifications of some one, more or less distinct, species; in other words, that we were dealing with a group, the individuals of which were related to each other in a way suggestive of the relation between the typical *Bacillus coli communis* and the numerous varieties that are embraced within the so-called colon group of bacilli. For the sake of convenience, various attempts were made to classify them into sub-groups according to more or less conspicuous peculiarities, but in no instance has it been possible to draw sharp dividing lines.

With regard to pathogenesis: Because of the resemblance of these organisms to *Vibrio metchnikovi*,* with which we are strongly inclined to think them either identical or very closely allied, the pigeon was employed as the test object for their disease-producing powers. By this test we could readily divide them into two primary groups, viz. pathogenic and non-pathogenic, though many of the members of these two groups have a number of cultural characteristics in common. Many of those found to be pathogenic are but feebly so, and many of those found to be non-pathogenic possess most of the cultural characters of the fully and feebly pathogenic ones, indicating the probability of their being but attenuated specimens of the pathogenic group. We have been obliged, therefore, to subdivide these two primary groups according to their disease-producing properties for pigeons as follows:

1. Those that are distinctly pathogenic, causing death of the animal

* See *The Journal of Experimental Medicine*, i (1896), p. 427 et seq.

and producing constant pathological lesions after a more or less fixed interval of time.

2. Those that are feebly pathogenic. These may or may not cause death of the animal, and the lesions resulting from their action upon the tissues are atypical.

3. Those that are non-pathogenic, but which possess, in a conspicuous degree, the majority of the cultural characteristics of those embraced within groups 1 and 2, especially the production of indol in considerable amounts.

4. Those that are distinctly non-pathogenic, and which differ in several of their cultural peculiarities from members of the other groups.

The organisms embraced within the fourth group may, perhaps, be regarded as forming a distinct class, for the reason that they all failed to give the indol reaction when tested with sulphuric acid alone, though with few exceptions this reaction was obtained when they were subjected to the action of sodium nitrite and sulphuric acid; that is to say, they have the power to produce indol, but not to reduce nitrates.

The cultures of the first three groups showed more or less marked indol-production upon the addition of sulphuric acid alone; those of the first and second groups most decidedly so, while with those in the third group the reaction was variable, being quite marked with some, with others less decided. They all had, therefore, the power of reducing nitrates.

These organisms may be divided into two groups also according to the structure of their colonies in 10 per cent nutrient gelatin. The first group comprises those individuals whose deep colonies are distinctly rosette- or mulberry-like, and which rapidly liquefy the gelatin, on coming to the surface, in the form of a funnel- or cup-shaped depression. The margin of these liquefying colonies is homogeneous, and their surface finely granular. The organisms forming the second group possess somewhat variable cultural features. The deep colonies are less distinctly rosette-shaped, and sometimes show thread-like bands that interlace over the surface and around the margin. After liquefaction has set in, the margin of the colony is less homogeneous than in those of the first group, the surface is coarsely granular, and masses of the colony are to be seen floating in the liquefied pit.

If we attempt to classify them according to some of their other biological characters we are able to group them in several different ways; yet none of the groups so formed are very definite, and most of the individuals in one group are different from those allied with them under some of the other modes of grouping.

With regard to their growth on potato we may classify them into three groups: (1) those growing luxuriantly on potato; (2) those growing only sparingly; and (3) those showing no visible growth.

With regard to their development on Lœffler's serum mixture they fall into four groups: (1) those that rapidly liquefy the serum; (2) those that slowly liquefy it; (3) those in which liquefaction is so slight as to be doubtful, and (4) those that cause no liquefaction.

As regards indol-production it is possible to classify them into five groups: (1) those that show marked indol-production on the addition of sulphuric acid alone; (2) those that show feeble indol-production on the addition of sulphuric acid alone, but in which the color-reaction becomes very much intensified on the further addition of sodium nitrite; (3) those that show no reaction on the addition of sulphuric acid, but show marked reaction on the further addition of sodium nitrite; (4) those that show no reaction with sulphuric acid alone and only a slight reaction with sulphuric acid and sodium nitrite, and (5) those that fail to reveal the presence of indol when treated with both the acid and the reducing body.

With a few exceptions the individual members of the several groups formed in either of these ways are not identical in all respects. The type of organism most frequently encountered is identical with the vibrio that formed the topic of last year's communication, and at the time was designated as *Vibrio schuylkiliensis*.*

The other organisms differed from this type in one or more characteristics. With some the variation is quite insignificant, with others it is more marked; while those that are most unlike this type still possess features which are common to the type, and in this way render definite grouping impossible.

The morphological characters of the different organisms also vary

* *The Journal of Experimental Medicine*, i, 419.

somewhat. They vary as to length and thickness, and also with regard to the length of spirals formed. Some of them rarely occur as other than short "comma" forms, while others exhibit a tendency to develop as spiral threads comprised of three or four single commas joined together. Others are prone to grow into much longer spiral threads in old cultures. These morphological tendencies afford no assistance in our efforts at classification, because those that are alike from this standpoint are frequently unlike in their other peculiarities.

We have also endeavored to detect differences between these cultures through the employment of the specific reaction that occurs between certain pathogenic bacteria and the serum from animals infected with or artificially immune from them. For this purpose animals were rendered immune from several of the more conspicuously pathogenic members of the group, and the serum from them was tested upon the various cultures by the ordinary hanging-drop method now employed in connection with the diagnosis of typhoid fever. As far as these experiments have gone, we have not obtained a complete reaction, as indicated by agglutination of the individual bacteria and cessation of motion; the nearest that we have been able to come to this has been agglutination with only partial cessation of motion. The reason for this may be that our animals were not brought to a sufficiently high state of immunity, being protected from only about ten times the fatal intraperitoneal dose. At all events, the serum from such animals (guinea-pigs), as well as the serum from animals in the height of acute infection, did not in any case give us what we would regard as a typical and satisfactory reaction.

Two of the cultures that were pathogenic, but which from their cultural peculiarities might be regarded as less alike than the remaining members of the group, were used to immunize guinea-pigs. After bringing the animals to a stage of immunity in which they resisted about ten times the fatal intraperitoneal dose, the serum from the one, immune from what we shall call type *a*, gave the partial reaction with a large proportion of the cultures; while the serum of the animal immune from the type *b* gave the partial reaction with but a very small number of the cultures. The serum from the animal immune

from type *a* gave no reaction with type-culture *b*, nor did the serum of animal *b* react with type-culture *a*. On the other hand, a number of the cultures reacted to some extent with both serums, and a smaller number were unaffected by either serum, though with one exception the cultural peculiarities of these latter were essentially identical with those that gave the reaction.

As a result of our efforts to differentiate these cultures by further experiments upon animals, we have found that when guinea-pigs were rendered immune from a single more or less typical member of the group, they were at the same time immune from five times the fatal dose of all the other pathogenic varieties.

In the light of the foregoing differential tests we are inclined to regard all the cultures obtained by us as varieties of a single species, the important characteristics of which are those given for the spirillum described by one of us (A. C. A.) in the paper published in the preceding volume of this journal.* The apparent absence of certain physiological functions, such for instance as that of indol-formation and nitrate-reduction, we do not, under the circumstances, regard as of the same degree of diagnostic importance as was formerly the case, for in the light of the experiments of Dr. Peckham, published in this number of the Journal, we know that these functions are liable to fluctuation, and that by special methods of cultivation they may often be restored to organisms from which, under the influence of environment, they were temporarily lost or very much diminished in degree.

The interesting and important outcome of these experiments seems to us to be the demonstration in the waters examined of spiral forms possessing characteristics that might readily lead one to mistake them for the genuine cholera spirillum, should the existence of suspicious intestinal disorders in the neighborhood appear to warrant such an opinion.

Many of the spirilla obtained by us are of the usual size and shape of the cholera organism; they produce indol and reduce nitrates; their colonies are sufficiently like those of the cholera organism to easily cause error; they are often without pathogenesis for pigeons when inoculated

* Loc. cit.

subcutaneously, a property common to the comma bacillus, while intraperitoneal inoculation of guinea-pigs is fatal, and death is preceded by the gradual fall of body temperature that is seen to occur in these animals when inoculated in this way with virulent cultures of the cholera spirillum; and like the latter organism our spirilla are not pathogenic for rabbits.

While the pathogenesis of the typical members of this group for pigeons at once distinguishes them from the spirillum of Asiatic cholera, the absence of this property from many of the attenuated varieties, which at the same time often retain the property of infecting guinea-pigs by intraperitoneal inoculation, when taken together with the striking similarity between their general biological peculiarities, renders a mistake quite possible.

It is our opinion that the only trustworthy distinction that can be made between many of these varieties and the genuine cholera spirillum is through the employment of either the specific reaction with serum from animals immune from cholera, or by Pfeiffer's method of intraperitoneal testing in such animals. These latter methods have not been employed by us.

The desirability of more extended knowledge of the nature and distribution of the spirilla in our American surface streams we hope, by these studies, to have made manifest.

# A MODIFICATION OF THE METHOD FOR DETERMINING THE PRODUCTION OF INDOL BY BACTERIA.

By THEOBALD SMITH, M. D.
(*From the Laboratory of Comparative Pathology, Harvard Medical School.*)

The Dunham-Bujwid indol test, used originally to distinguish cholera spirilla from other spirilla, was applied by Kitasato to bacteria other than those of Asiatic cholera, notably the colon bacillus and the typhoid bacillus, and to-day the test for indol is, by common consent, used regularly in the study of bacteria. As a result of its application we now know of two classes of indol-producing bacteria—those which produce, in addition, nitrites, and those which do not. The first includes the spirillum of Asiatic cholera; the second, most other indol-producing species. The fairly extensive literature deals mainly with the first class, because of the obviously important bearing of any method which will distinguish cholera from other spirilla. As is well known, the "cholera-red" reaction is brought out by adding sulphuric or chlorhydric acid free from nitrous acid to bouillon cultures, when the presence of indol plus nitrites will impart a violet-red color to the fluid. Dunham introduced the nutritive solution consisting of 1 per cent peptone and ½ per cent sodium chloride in water. This is now universally used in testing for indol.

There have been not a few voices raised in criticism of this method for determining the cholera-red reaction. To these I shall refer only as they antedate my own efforts towards improving the indol test, since I have restricted myself to the purely indol-producing forms. In attempting for a number of years past to apply the indol test by using the simple peptone solution I have been forced to consider the method quite unsatisfactory for some groups of bacteria. I was therefore led recently to try dextrose-free bouillon, with a premonition that possibly the carbohydrate may inhibit the production of indol as it does that of toxins in some cases. The result was very satisfac-

tory. Other facts were brought out at the same time which tend to explain the failure of the test under certain conditions. A search through the literature showed me that the restraining influence of sugar had been already demonstrated. Gorini,* in discussing the uncertainty of the cholera-red reaction, states that in the presence of 0.5 per cent dextrose, saccharose and lactose, cholera spirilla fail to give the red reaction. He lays the blame upon the presence of traces of sugar in the peptone and gives a number of tests by which a pure peptone may be recognized. Kruse† briefly refers to this subject by stating that when bouillon contains more than 0.25 per cent sugar the indol reaction no longer appears. He adds that cultures should remain at least a week in the thermostat, a point brought out in the experiments to be given below. These are the only references I can find which bear directly upon my own observations. These I shall very briefly summarize.

1. The peptone‡ solution is a very poor culture fluid for many bacteria; some fail to grow in it. Dextrose-free bouillon is far better. All bacteria tried grew in it, some quite vigorously. In the dextrose-free bouillon the indol reaction is pronounced in 16-hour cultures, while in peptone solution it required from three to six days for traces of indol to form. The coloration of the dextrose-free bouillon becomes intense, usually within a few seconds after the nitrite and acid are added. In the peptone solution it remains feeble, often so much so that a decision is impossible.

2. In bouillon containing muscle sugar the indol reaction becomes positive after the bacteria have converted the sugar into acid and nearly neutralized the latter by alkali production. If the bacteria are unable to neutralize the acid formed, either because too much sugar was present or because they are inherently unable to produce much alkali, the indol reaction will fail. This is well illustrated by two indol-producing groups, the colon and the rabbit-septicæmia group, the former a rapid, abundant alkali producer, the latter not.

* Anmerkung ü. d. Cholerarotreaktion. *Centralbl. f. Bakt.*, xiii (1893), 790.

† Kritische u. exp. Beiträge z. hygienischen Beurtheilung d. Wassers. *Zeitschr. f. Hygiene*, xvii (1894), 1.

‡ That used was from two lots of Witte's peptone.

Comparative experiments were made by inoculating a series of tubes, one a day, for eight or nine days. All were tested for indol at the same time, so that the time element in the formation of indol might not be neglected. Each tube (cotton-plugged) received about 1 cc. of a 0.01 per cent solution of $KNO_2$ freshly prepared and 10 drops of chemically pure $H_2SO_4$.

With B. coli in a bouillon containing enough muscle sugar to greatly inhibit the multiplication of diphtheria bacilli, the indol reaction appeared intensely after the acid formed had been sufficiently neutralized by alkali production to give a feebly blue color with neutral litmus, or when the acidity, according to phenolphthalein, was about 1 per cent of a normal solution. This point was reached on the third or fourth day. Younger cultures gave only a feeble tint. On the other hand a culture of fowl cholera bacteria (rabbit-septicæmia group) in bouillon containing muscle sugar failed to give any indol reaction in any tube of the series. The reaction remained acid to litmus. The same culture gave an intense violet-red color in dextrose-free bouillon after 18 hours in one series, 48 hours in another.

When Dunham's solution was used the fowl cholera bacteria gave a feeble but easily recognizable tint in the tube 9 days old, but none in the 7 younger cultures. The colon bacillus gave a well-marked violet-red color in tubes older than 3 days, no distinct change in those younger than 3 days. In dextrose-free bouillon a deep violet-red was brought out in 18-hour tubes, growing more intense in cultures according to their age. Tubes less than 18 hours old have not been tried.

Cultures of B. coli in the fermentation tube were also tested. In former publications I have pointed out that while the entire fluid becomes acid when sugar is present, the fluid in the closed branch remains so, and that in the open bulb in free contact with oxygen becomes alkaline provided the sugar does not exceed a certain amount (0.3 to 0.4 per cent, approximately, for B. coli). In such cultures the acid fluid in the closed branch gave a negative, the alkaline fluid in the bulb a positive indol reaction.*

* Since the acid reaction remains while the anaerobic conditions persist, it would seem as if indol were not producible by facultative-anaerobic bacteria, such as B. coli, except in contact with oxygen, unless the method of demonstrating indol, here used, is at fault.

3. From what precedes it is evident that mere traces of dextrose in peptone cannot be held responsible for the feeble indol reaction, as claimed by Gorini. A two per cent peptone solution tested in the fermentation tube showed only the faintest growth in the closed branch when inoculated with B. coli. It is furthermore evident that sugars inhibit indol production only under certain conditions, varying with the organism studied. The advantages of the dextrose-free bouillon over the peptone solution may be due either to its greater general nutritive value or to the presence of substances yielding indol more readily than does the peptone.

The preparation of dextrose-free bouillon is simple enough. Some years ago the writer selected such bouillon from that made in the ordinary way by testing every lot in the fermentation tube. This method is unsatisfactory, because most beef contains more than traces. Spronck's method, of allowing beef to decompose for several days, is unreliable because the bouillon made from it frequently contains relatively much sugar. The procedure now used by me is uniformly successful. Beef infusion, prepared either by extracting in the cold or at 60° C., is inoculated in the evening with a rich fluid culture of some acid-producing bacterium (I use temporarily B. coli) and placed in the thermostat. Early next morning the infusion, covered with a thin layer of froth, is boiled, filtered, peptone and salt added and the neutralization and sterilization carried on as usual. Such bouillon when tested with B. coli in the fermentation tube will no longer permit any anaerobic growth. The closed branch remains clear, a sign that carbohydrates are absent.* It might be claimed that such bouillon would normally contain indol. This is not the case. Its absence was shown by the negative indol reaction of the sterile bouillon and by the negative indol reaction of bacteria, such as typhoid, hog cholera (several varieties), mouse septicæmia, B. lactis aerogenes and diphtheria,† of which series were tested as detailed above.

* T. Smith. Ueber d. Bedeutung des Zuckers in Kulturmedien f. Bakterien. *Centralbl. f. Bakt.*, xviii (1895), 1.

† Contrary to Palmirski and Orlowski (*Centralbl. f. Bakt.*, xvii (1895), 358), who claim that the diptheria bacillus produces indol.

Nitrites were likewise absent, for the indol-producing bacteria did not give the indol reaction when $H_2SO_4$ alone was added.

Questions bearing on the production of indol in the presence of different kinds of peptones, and of sugars not acted upon by the bacteria under examination, as well as in bouillon free from peptone, are left untouched. The effect of the change of procedure upon the cholera-red reaction is likewise abandoned for the present, because cholera cultures are not available.

# THE INFLUENCE OF ENVIRONMENT UPON THE BIOLOGICAL PROCESSES OF THE VARIOUS MEMBERS OF THE COLON GROUP OF BACILLI.

## An Experimental Study.

By ADELAIDE WARD PECKHAM, M. D.
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The results found in the following pages have been made possible by a grant from the Bache fund of the Smithsonian Institution. The disposal of the grant mentioned is left to the discretion of Dr. John S. Billings and Dr. S. Weir Mitchell. The topic of this research was submitted to these gentlemen at the beginning of the year, and from time to time they have been kept informed of the progress of the work. It is with their approval that this paper is presented for publication. In the autumn of 1894 this fund became available for our research, and a study was begun upon the influence of light, color, desiccation, etc., upon the vitality of the colon and typhoid bacilli and of the staphylococcus pyogenes aureus. The details of this research were published in *Science,* February 15, 1895. The following year the research was continued, the subject being upon the variability of bacteria as shown by modifications of function in different varieties of the colon group. The cultures studied were obtained from sources where the micro-organisms present might be expected to show retrogressive changes; the results of this year's study were also published in *Science,* November 27, 1896. During the last academic year this research has still been continued, and our time has been devoted principally to the study of methods for enhancing the proteolytic activity of the various members of the colon group.

As is the case with the more highly organized members of the animal and vegetable world, bacteria experience modifications of their physiological functions according as they are exposed to the influence of various circumstances. In some instances the environment may favor an excessive activity of all functions, in others it may cause their depression, while again under other surroundings one function or

group of functions may be accentuated while others are more or less suppressed.

Of the factors that are conspicuous for their influence upon the biological activities of bacteria, food is one that is manifestly of fundamental importance. In the selection of those substances most suitable for the nutrition of bacteria, each class of food must be considered in regard to the form in which it is best assimilated by micro-organisms, and to the proportion which is necessary for their most vigorous growth. Plastic material, of which the cell body is composed and by which growth, reconstruction and reproduction are made possible, is derived from the proteid bodies; for the compensation of energy and apparently for the maintenance of the gaseous respiration of the cells, the carbohydrate class of foods is essential. These latter undergo, as a result of the life processes of the micro-organisms to which they are supplying essential nutritive materials, certain chemical decompositions, of which fermentation and oxidation are the most frequent and most easily recognizable.

As we shall learn from experiments shortly to be described, there are additional analogues between the life functions of unicellular and of complex organisms. We shall learn that the life of the organism as a whole is strikingly influenced by growth under conditions that, as in our case, favor excessive development of a particular function, or that permanently suppress certain other life processes. Bearing upon this particular point, Spencer, in discussing the functions of man, makes reference to what from our observations seems equally true of the functions of single cells. He remarks that "every function has some relation, direct or indirect, to the needs of life." "If there is defective discharge of some function, the organism experiences some detrimental result caused by the inadequacy. If the discharge is in excess, there is entailed a reaction upon the other functions which in some way diminishes their efficiencies." "If some function is performed in excess or defect there results some chronic derangement in the balance of the functions. Death is more or less premature."

An especially interesting group for the study of cellular physiology and for the observation of deviations in function is the colon group of

bacteria, the members of which present close similarities morphologically, but vary considerably in regard to important biological processes.

The most characteristic activity of this group is that of fermentation, either gaseous or otherwise, of the carbohydrates, which appears to be common to all its members. By this process glucose is split into lactic acid through the action of an enzyme, which is one of the products of metabolism in these and many other micro-organisms. The formation of lactic acid completes the first stage of fermentation; and in some species of this group, as in the typhoid bacillus, the process ceases at this point. In many, however, the lactic acid is broken up by a process of hydration into less complex substances, mostly gases, and this step forms the second stage of fermentation.* The fermentation of lactose and saccharose varies considerably in different members of this group, because only a part of them produce an inverting enzyme by which they are enabled to split these condensed glucoses into ordinary glucose, which in turn is broken up by the lactic acid enzyme.† The influence of invertin in this group is very slow, probably because these bacteria cannot endure the conditions that are most favorable to its action, viz. a temperature of 56° C. and a considerable degree of acidity of the medium.

The following details relating especially to the function of gaseous fermentation, as exhibited by the group of colon bacilli, have been condensed from the elaborate studies of Theobald Smith (1). All of this group, which includes many of the species which are closely related to the colon and typhoid bacilli, ferment dextrose, as shown by the production of acid, and many times by the production of gas. Many species ferment lactose and saccharose, but the following variations concerning their action may be noted: First, there are species which invert lactose and saccharose into glucose and complete the process of

* The decomposition of glucose by the lactic acid enzyme is represented by the following equation:

(1) $2C_6H_{12}O_6 + \text{enzyme} = 4C_3H_6O_3$.

(2) $C_3H_6O_3 + 6H_2O = 3H_2CO_3(3H_2O + 3CO_2) + 12H$.

† The conversion of lactose and saccharose into glucose is represented as follows: $C_{12}H_{22}O_{11} + H_2O + \text{invertin} = 2C_6H_{12}O_6$.

fermentation by the production of gas. Secondly, some species invert lactose and saccharose and pass through the first stage of fermentation, namely, the production of acid. Thirdly, others cannot invert these sugars and therefore cannot produce fermentation. Fourthly, some act upon one of these sugars and cannot split the other.

The observations upon fermentation that we shall detail in this contribution have been made upon cultures grown under such conditions as would be liable to cause alterations in function.

A still more variable function of this group is its action upon proteid substances. These bacteria do not produce a proteolytic enzyme, and therefore are dependent upon some means external to their cell bodies by which the various forms of proteids can be converted into substances suitable for their assimilation, that is, into peptones or albumoses or some one or another of the albuminoid molecules that can easily pass through their cell membrane. The peptonizing ferments of animal life may be brought into requisition for this purpose, or the enzyme of other micro-organisms may be utilized.

Fermi and Pampersi (2), in their research upon the peptonization of proteids by micro-organisms, conclude that the albumin prepared for assimilation by one microbe does not necessarily enter that cell, but may be appropriated by another which is present. They found further that the proteolytic enzyme is not peptogenic; it does not produce peptones, and the micro-organisms are not dependent upon them. Albumin is transformed in the same manner as it is in the walls of the intestine.

Proteid digestion can be approximately estimated in many members of the colon group by the production of indol, whose peculiar reaction permits of an easy approximate estimation of the quantity produced. Indol formation, however, varies greatly in the different members of this group, and varieties are well known that do not appear to produce it, while others possess that power to a marked degree. Cultures obtained from river water and other sources where proteid material is sparingly found may become so changed by long deprivation of suitable proteid food that they apparently lose this indol-producing function. It may be that the last stage of the proteolytic

process, like gas production in the last stage of fermentation, is soon modified or lost by agencies which in general induce an attenuation of micro-organisms. Many cultures which do not produce indol exhibit analogous deviations in other functions, that may be accounted for by lack of uniformity in the degree of their vital activities, rather than by regarding them as distinct species.

Cultures which we obtained by a process of attenuation, and also others which we recovered from the organs of animals inoculated with a tested culture, and also from the spleens of persons dead of typhoid fever, show the above-mentioned variations in function and especially in fermentation. A study of the colon group in a proteid medium was suggested by such results, and forms the theme of this article. This subject has been extensively investigated, but the results that have been obtained do not appear to have attracted the attention that their importance should warrant.

Péré (3), in a research on the formation of lactic acid by micro-organisms, found that the same microbe may produce lactic acids of opposite optical activities, according to the quality and quantity of the proteid material used. The amount of acid formed diminishes when the medium is rich in peptone, even when the ratio of dextrose remains constant.

Fermi and Salto (4), in a study of the intestinal canal in its relation to the immunity of certain animals from the cholera vibrio, state that the greater the pathogenic power of the colon bacillus the less prominent are its saprophytic properties, for instance, the fermenting power.

The effect of proteid material upon the virulence of the colon bacillus has been often observed, not with reference to artificial cultivation, however, but in cultures obtained under conditions where proteid substances were increased by various inflammatory conditions in animals or man.

Klecki (5), Sanarelli (6) and Dreyfuss (7) conclude from their researches that the pathogenesis of the colon bacillus is acquired by growth in albuminous material which is afforded by the products of inflammation.

The nutritive value of the original proteids, casein, muscle, egg albumin, blood serum, etc., for bacteria has been considerably studied in various culture fluids. Péré, in 1892, published a most interesting and instructive article upon the bacillus coli communis and the bacillus typhosus, relating to their growth upon different forms of proteids peptonized

by different ferments. The suggestions afforded by his results have been utilized in our study of the colon group.

He found that indol production is greater with a pure peptone solution than with any other form of proteid; the dried peptone, however, gives comparatively little indol. Furthermore, the colon bacillus forms indol with proteids peptonized by each of the various proteolytic enzymes, even with the vegetable pepsins, but trypsin forms a peptone which gives the most indol. Peptones prepared by digesting casein with trypsin give a much larger amount of indol than acid or alkali albumin, or than acid peptones prepared by the action of pepsin.

Deycke (9) advocates the use of an alkali albumin in media employed for special purposes. He uses a solution containing two to three per cent of alkali albumin as an elective medium for the diagnosis of cholera cultures, in which the colonies develop quickly and luxuriantly, while other organisms* contained in the feces are inhibited. Later he found that this medium is useful for detecting the bacillus diphtheriæ.

We have used culture fluids containing peptones derived from various proteids through the action of pepsin and trypsin, and have obtained the best results from those prepared from beef muscle with trypsin. These peptones differ from those of Péré by being derived from fresh muscle, while his were obtained from casein. Both differ from Deycke's in that they contain true peptones and albumoses, obtained in alkaline mixture, while Deycke's alkali albumin is derived from a proteid through the action of a dilute alkali, without peptonization. Our medium is called throughout this article alkali-peptone bouillon, and was prepared by the following formula:

Beef muscle, 225 grammes.
Trypsin, 4 grammes.
Salt, 5 grammes.
Water, one litre.

The finely chopped beef muscle, which must be as old as can ordinarily be obtained in shops, in order that it may be free from muscle sugar, is put into 500 cc. of water and the mixture made slightly alkaline with sodium carbonate. The flask containing the

* The colon bacillus does not thrive upon alkali albumin because it does not produce the enzyme necessary for converting alkali albumin to peptone.

mixture is then placed in a water bath at a temperature of 40° C. and the trypsin is added. After digesting for an hour the fluid becomes decidedly acid. This is probably in part due to the sarcolactic acid set free from the tissue, and in part to free carbonic acid resulting from decomposition of the carbonates. The fluid must again be made alkaline by the addition of sodium carbonate. Our experience indicates that, in general, peptonization should be arrested in from one to one and one-half hours, otherwise traces of indol may be detected. At the end of this period boil the mixture and strain through gauze and then filter cold through wet filter paper to remove the fat; then add the salt and enough water to make one litre. The acidity of the clear, straw-colored filtrate is then reduced to the desired point, to be given hereafter; the indicator used is phenolphthalein. After this it is clarified, filtered and sterilized. After sterilization the bouillon is tested for indol and sugar, the latter test being made with Soldaini's cupric solution. If these undesirable products are present the indol, of course, prevents exact estimation of the amount formed by the organisms under study, and the sugar interferes with the action of the bacteria upon the proteid substances. We have experienced no great difficulty in obtaining meat free from sugar. The bouillon is also tested for peptones by the biuret reaction, which is compared with the same reaction in a one per cent dried peptone solution.

Different observers have found a slightly alkaline medium to be favorable to the growth of many bacteria. Hesse (10) tested the vibrio of cholera in a medium to which varying quantities of sodium carbonate were added and found that the addition of 0.5 cc. of a normal solution of sodium carbonate to 25 cc. of agar favored the growth of the spirillum. Blumenthal (11) also studied the influence of an alkali on the activities of microbes, especially on their chemical products. He concludes that the formation of putrefactive products depends on the *alkalinity of the material decomposed* and not on an alkali added to the medium. The virulence of the colon bacillus was increased, however, by adding an alkali to the bouillon used as a medium.

The reaction most desirable for the colon group of organisms must

depend, however, upon the constituents of the medium. In a medium containing sugar a slight alkalinity is desirable to neutralize any excess of acidity due to the fermentation of the sugar; but in the absence of fermenting material this organism breaks up peptones and thereby progressively increases the alkalinity. The influence of albumin on the reaction of media must, then, be taken into consideration in deciding the reaction of media rich in proteid material. Timpe (12), in an article relating to this subject, emphasizes the fact that because all albuminous bodies give an alkaline reaction to litmus, its use as an indicator in a peptone-holding medium makes the alkaline reaction prominent, while a considerable amount of free acid is in reality present; and because albuminous bodies give an acid reaction to phenolphthalein, the use of this indicator increases the alkalinity of the medium, making a sharp neutralization possible.

Our experience teaches that a medium containing such an amount of free acid as to require from 20 to 30 cc. per litre of a decinormal caustic soda solution to bring it to a point neutral to phenolphthalein is the most suitable reaction for the development of this organism when sugars are not present.

There is an interesting but somewhat confusing array of evidence bearing upon the development of pathogenesis by the colon bacillus. We will review briefly the observations and deductions of different investigators, and finally relate the results of our own experiments with different species of the colon bacillus, both *in vitro* and in animals.

Klecki (5), in his experiments upon dogs, found that the colon bacillus acquires virulence in the lumen of a strangulated intestine. This virulence, he concludes, is the result of development in the large amounts of albuminous material which collect in the loop of intestine. The virulence of the bacilli which pass through the intestinal walls into the cavity of the peritoneum is also increased, but not to the same degree as in those in the intestine.

Dreyfuss (7) found that cultures of the colon bacillus obtained from normal feces are non-pathogenic, but in intestinal diseases they become virulent to an extent which is in proportion to the severity of the disease.

Fermi and Salto (4) conclude that any abnormal condition of the intestinal mucous membrane, such as would be produced by ligatures,

cold of the abdomen, too much food, etc., increases the virulence of the colon bacillus.

Sanarelli (6) caused the colon bacillus to become virulent in experimental typhoid fever by producing an inflammation with the typhoid toxin.

All of these writers appear to favor the theory that the virulence of the colon bacillus is the result of growth in the living fluids of the body, which are supplied with an unusual amount of albuminous matter by the process of inflammation. The seat of the inflammation, which may be either in the intestine or in other viscera, influences to some extent the degree of virulence.

What constitutes the difference in the conditions existing in the intestine in health and disease, which causes the colon bacillus to be non-virulent in one case and virulent in the other?

The normal intestine provides food in a form most favorable for assimilation by this organism and from which it forms the most indol, namely, peptone, the product of the tryptic enzyme. There is also supposed to exist a commensal relation between the cells of the intestinal mucous membrane and the colon bacillus. But it does not become virulent even under these favorable circumstances, for the reason, as we shall attempt to show in this paper, that its first force is spent upon the process of fermentation, and as long as opportunities exist for the exercise of this function the affinities of this organism appear to be strongest in this direction. Moreover, the contents of the intestine remain acid until they reach the neighborhood of the colon, and by that time the tryptic peptones have been formed and absorbed to a great extent. Under such conditions the colon bacillus has relatively little opportunity to assume an excess of proteolytic activity.

During the process of inflammation in the digestive tract a very different condition may exist. The peptic and tryptic enzymes may be partially suppressed. Fermentation of carbohydrates and proteid foods then begins in the stomach and continues after the mass of food is passed on to the intestine. The colon bacillus cannot, therefore, spend its force upon fermentation of sugars because they are already

broken up and an alkaline fermentation of the proteids is in process. It also cannot form peptones from the original proteids, for it does not possess this property, and unless trypsin is present it must be dependent upon the proteolytic activity of other bacteria for a suitable form of proteid food. Perhaps these bacteria form an albuminoid molecule, which, like leucin and tyrosin (13), cannot be broken up into indol, and thus there might be caused an important modification in the metabolism of the colon bacillus which may have either an immediate or remote influence upon its acquisition of disease-producing properties, for our own experiments indicate that the power to form indol and the actual forming of it are to some extent an indication of the possession of pathogenesis.

In our experiments upon the colon bacillus with a medium characterized by large amounts of freshly formed peptones and entire absence of fermentable material, we find that the proteolytic function is greatly increased in activity, as measured by the amount of indol resulting from its growth. If, however, the cultivation be carried on beyond the period of maximum indol formation, and each generation be restricted to this specific activity by being transplanted to fresh media of the same character as soon as the growth reaches its maturity (3 days), a modification of the vital activities of the organisms occurs. Indol formation is gradually abolished, even though it may have been excessive at an earlier period, and the culture finally dies prematurely.

The colon bacillus apparently soon dies when it is introduced into the peritoneal cavity of guinea-pigs, even if the amount injected is sufficient to set up a fatal inflammatory process. In seventy-two guinea-pigs inoculated intraperitoneally with the colon bacillus the organism was recovered from the different organs of only the animals that died by the fourth day. After that period the peritoneum and organs were sterile. The cultures obtained from these animals after inoculation showed, even after this length of time, a slight modification in certain important functions, notably that of fermentation.

In certain members of the colon group which possess less vital activity than does the typical colon bacillus, as shown by a disturbance

of functional equilibrium, recognizable by a partial or complete loss of either the proteolytic or fermenting function, cultivation in a medium containing a large amount of peptones gave similar qualitative results, though the time necessary for the results was much longer than was that required for the typical colon bacillus. Proteolytic action as indicated by indol formation was at first entirely absent in all of these cultures. In this case the frequent change to a fresh medium increased the vital activities for several generations, until a marked indol reaction was obtained which seemed to be the maximum amount that these cultures were able to form; then, as with the typical organism, degeneration and death of the cultures took place. But, unlike our experience with typical cultures, this acquisition of the indol-producing function was very much more gradual, so that the maximum of the indol curve is conspicuously retarded (see graphic representation in accompanying chart, p. 561).

*Indol tests.*—A preliminary indol test was first made to determine at what age a culture of a typical colon bacillus inoculated into a peptone solution gives the greatest amount of indol. Typical cultures of the bacillus coli communis were tested, ten tubes of each culture being inoculated at the same hour into the alkali-peptone bouillon. One tube was tested for indol at the end of each twenty-four hours. A large number of tests made at the end of the intervals named proved that indol production nearly or quite ceases in seventy-two hours after inoculation. All of the subsequent comparative tests were made, therefore, on cultures of that age.

TYPICAL CULTURES OF THE BACILLUS COLI COMMUNIS TESTED FOR INDOL.

A series of ten typical cultures was inoculated into the alkali-peptone bouillon and also into Dunham's dried-peptone solution, for the purpose of estimating the amount of indol formed and the time at which the maximum of indol production is reached.

Twenty-five cubic centimetres of each medium was placed in Erlenmeyer's flasks, which were inoculated at the same hour and placed in the incubator, where they remained until the fourth day. Transplantations were then made to fresh flasks containing a like medium, and the old cultures were tested for indol in the following manner: To 25 cc. of this

fluid culture, slightly warmed, both a solution of sodium nitrite (0.01 per cent) and of sulphuric acid (25 per cent) were added in varying proportions. The reason for our not having employed them in uniform rates is that we found with different cultures, under different circumstances, the maximum amount of indol could only be decided by corresponding variations in the proportions of nitrites and acid used. For instance, we observed that frequently decided evidence of the presence of indol was manifest by the addition to 25 cc. of the culture of 1 cc. of sulphuric acid and 1 cc. of the nitrite solution, but this by no means represented the greatest degree of reaction obtainable in this culture; by the further addition of both acid and nitrite solution the density of color of the indol reaction was very frequently appreciably increased. Whether or not this increase of density in the red color in reality indicated larger amounts of indol we cannot say certainly, but from control experiments made with varying but known weights of indol we are inclined to the opinion that it does.

The method that we have employed for estimating the varying amounts of indol produced is not regarded by us as in every way satisfactory, but the results obtained by its use, we think, approximate sufficiently closely to accuracy for all practical purposes of comparison. The principle of the method is similar to that of all colorimetric tests, that is to say, standard solutions of varying and known strengths of pure indol (as obtained from the manufacturers) were prepared, placed in Nessler tubes, and the usual reaction made. In similar tubes our culture, in which the reaction had also been made, was placed, and its tint compared with those of the standards. The procedure in detail as carried out by us is as follows: Nessler tubes (30 cm. long, 1 cm. in diameter) filled with the indol solution are prepared by adding one-tenth of a cubic centimetre of a one-tenth per cent solution of indol, to which enough acid and nitrite have been added to give the nitroso-indol reaction, to enough water to make 50 cc. The reaction for indol is then made in the culture under consideration, after which varying amounts of this culture are placed in similar Nessler tubes and the volume of each is brought up to 50 cc. The tints of these dilutions are then compared with the standards, and the approximate amount of indol in the culture is determined from the result, just as it is done in the ordinary process of nesslerization for ammonia.

TABLE I.

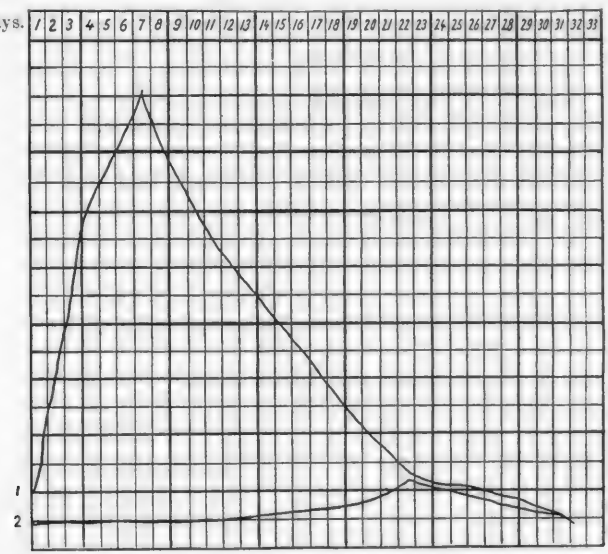
SHOWING THE PERIOD OF INDOL FORMATION OF CULTURES OF THE TYPICAL BACILLUS COLI COMMUNIS WHEN CARRIED THROUGH A SERIES OF TRANSPLANTATIONS IN PEPTONE SOLUTIONS. THE LIFE OF EACH GENERATION WAS 3 DAYS.

The figures in the columns indicate the amount of a one-tenth per cent. indol solution, expressed in cubic centimetres, which was required to give the same tint as the indol formed by the culture in 25 cc. of the medium.

| No. of culture. | Dunham's solution. | | | | | | Alkali-peptone bouillon. | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1st gen. | 2nd gen. | 3rd gen. | 4th gen. | 5th gen. | 6th gen. | 1st gen. | 2nd gen. | 3rd gen. | 4th gen. | 5th gen. | 6th gen. |
| 1 | + | .6 cc. | .3 cc. | .2 cc. | + | + | 10 cc. | 13 cc. | 10 cc. | 5 cc. | 3.2 cc. | .3 cc. |
| 2 | + | .6 cc. | .3 cc. | .3 cc. | + | + | 7 cc. | 13 cc. | 8 cc. | 4 cc. | 2 cc. | .3 cc. |
| 3 | + | .6 cc. | .3 cc. | .3 cc. | + | + | 6 cc. | 11.2 cc. | 7 cc. | 3.5 cc. | 1.3 cc. | .3 cc. |
| 4 | + | .6 cc. | .6 cc. | .3 cc. | + | + | 6 cc. | 11.2 cc. | 7 cc. | 4 cc. | 2 cc. | .3 cc. |
| 5 | + | .6 cc. | .6 cc. | .3 cc. | + | + | 8 cc. | 13 cc. | 7 cc. | 4 cc. | 2.5 cc. | .6 cc. |
| 6 | + | .2 cc. | .4 cc. | .4 cc. | + | + | | 13 cc. | 6 cc. | 6 cc. | 2.5 cc. | .5 cc. |
| 7 | .3 cc. | .2 cc. | + | + | + | + | 10 cc. | 11 cc. | 10 cc. | 6 cc. | 3.3 cc. | .3 cc. |

CHART SHOWING INDOL CURVE OF THE TYPICAL AND OF AN ATYPICAL COLON BACILLUS.

Each vertical space represents a cubic centimetre of the standard indol solution used for obtaining the same tint as the culture reaction, and each horizontal space represents an interval of 24 hours.




1. Indol curve of the typical colon bacillus.
2. Indol curve of an atypical colon bacillus.

The preceding Table I gives the results of the quantitative indol test upon the typical cultures. The test was repeated three times and gave slightly varying results. The amount of a one-tenth per cent indol solution which was required to give the same tint as the indol formed by the culture in 25 cc. of the medium is expressed in cubic centimetres. These cultures, which were all from old laboratory stock, reached the height of indol production in about seven days and in the second generation. The amount gradually lessened after that period. The difference in indol production of the same culture in the two solutions used, namely, Dunham's peptone solution and the alkali-peptone bouillon, is very marked. While usually a decided reaction is all that is obtained in the peptone solution, the alkali-peptone bouillon gives either a bright blood-red color or a darker purplish tinge.

ATYPICAL MEMBERS OF THE COLON GROUP TESTED FOR INDOL.

Cultures which varied from the typical colon bacillus in one or both functions were selected for this test. Nos. 1 to 8 inclusive are either transitional or pseudo-forms of the colon group. No. 9 is a culture of the bacillus lactis aerogenes and No. 10 is the bacillus cloacæ. These cultures have been frequently tested for indol by the usual method during the two years that they have been in our stock, and some of them have at times seemed to give a slight indol reaction.

This series, like the preceding, was inoculated into the alkali-peptone and Dunham's solutions and was carried systematically through several cultures in each medium. They were plated and examined microscopially to prevent contamination. The result obtained is tabulated in Table II. All except one of this series (No. 3) produced indol; but while the typical cultures were brought to the height of indol production in 7 days, the cultures of this series required from 14 to 20 days before giving a decided indol reaction. As in the preceding series, the reaction was less distinct in Dunham's solution than in the alkali-peptone bouillon. For this solution Sargent's dried acid peptone was used, because we have usually found it to be free from indol, while repeated tests on sterile solutions of different specimens of Witte's peptone have given positive reactions.

TABLE II.

SHOWING THE PERIOD OF INDOL FORMATION OF CULTURES OF ATYPICAL COLON BACILLI WHEN CARRIED THROUGH A SERIES OF TRANSPLANTATIONS IN PEPTONE SOLUTIONS. THE LIFE OF EACH GENERATION WAS 3 DAYS.

| No. of culture. | Dunham's solution. | | | | Alkali-peptone bouillon. | | | |
|---|---|---|---|---|---|---|---|---|
| | 1st gen. | 2d gen. | 3rd gen. | 4th gen. | 1st gen. | 2d gen. | 3rd gen. | 4th gen. |
| 1 | — | — | — | + | + | + | | |
| 2 | — | — | — | + | — | — | + | |
| 3 | — | — | — | — | — | — | — | — |
| 4 | — | — | + | | — | + | | |
| 5 | — | — | + | | ? | + | | |
| 6 | — | — | + | | ? | + | | |
| 7 | — | — | — | + | — | + | | |
| 8 | — | — | + | | — | ? | + | |
| 9 | — | — | + | | — | + | | |
| 10 | | — | + | | — | + | | |

RESULTS FOLLOWING THE EXCESSIVE DISCHARGE OF ONE FUNCTION.

A comparison of normal cultures with those in which there has been what to us seems an excessive discharge of one function shows that the latter finally undergo a modification in their products of metabolism that coincides with gradual loss of vitality and that they finally die prematurely.

*Excess of fermentation.*—Again regarding the bacillus coli communis as the type of this group, in which fermentation and proteolytic action are in normal proportion, we find that the former is the initial and characteristic function. All of the enzymes formed by this organism are designed to promote fermentation; and so natural is this function to this organism that even in solutions containing equal amounts of dextrose and peptone the property of fermentation always takes precedence over that of proteolysis, the proportion of indol formed having been regarded by us as a fair index of the activity of this latter function.

In media equally rich in dextrose and peptone the process of fermentation begins immediately and proceeds apparently alone, at least as far as indol production is concerned, until all of the sugar has been converted into lactic acid. Péré made observations in regard to this action in 1893 and stated that a positive reaction for indol proves the disappearance of sugar in the medium.

We have found by a large number of tests that the fermentation of lactose also, although requiring two enzymes, invertin and that of lactic acid, is entirely completed before indol formation begins; but in a one per cent saccharose bouillon indol is often present, while undecomposed sugar may still be detected in the medium by appropriate tests.

The height of acid formation in a medium containing the amounts of carbohydrates and proteids mentioned is reached at the end of 72 hours. At that time the acidity of the medium is sufficient to inhibit further activity, and later the bacilli fall to the bottom of the tube. The biuret test when applied to these cultures shows that a large part of the peptones remains unused, a further indication of the essential nature of the function of fermentation to the biology of this group of organisms and of the influence of the presence of sugars on proteid decomposition. This we regard as in part explainable through the exhaustion of energy during the breaking up of dextrose and not alone to the excess of acid formed; for we have found that when an amount of lactic acid equivalent to that formed in these cultures was added to the medium into which the above organisms were inoculated, that it did not entirely prevent the formation of indol.

The result, then, of excessive fermentation is the exhaustion of energy in the production of enough acid to inhibit the growth and finally to cause the death of the organism. The proteolytic function resulting in indol as an end product of metabolism is entirely abolished and the peptones are probably untouched, thereby causing a deficit in the amount of plastic material necessary to the building up of the bacterial cells.

TABLE III.

SHOWING THE EFFECT OF THE PRESENCE OF SUGAR UPON PROTEOLYSIS. THE CULTURES OF TABLE I, HAVING BEEN INOCULATED INTO A MEDIUM CONTAINING SUGAR AT THE TIME WHEN THEY WERE AT THE MAXIMUM POINT OF INDOL PRODUCTION, GAVE THE FOLLOWING RESULTS.

The figures in the columns indicate the amount of a standard indol solution used to give the same intensity of color as that given by the culture.

| No. of culture. | Alkali-peptone bouillon. | | Alkali-peptone bouillon containing ½% of glucose. | | Alkali-peptone bouillon containing ¼% of glucose. | | Alkali-peptone bouillon containing lactic acid. | |
|---|---|---|---|---|---|---|---|---|
| | Acidity. | Indol reaction. | Acidity. | Indol reaction. | Acidity. | Indol reaction. | Acidity. | Indol reaction. |
| 1 | 0 | 13 cc. | 18.5 cc. | trace. | 12.5 cc. | trace. | | — |
| 2 | 0 | 13 cc. | 17.5 cc. | trace. | alkaline. | 1 cc. | | 3 cc. |
| 3 | 0 | 11.2 cc. | 16 cc. | — | alkaline. | 1 cc. | | 3 cc. |
| 4 | 0 | 11.2 cc. | 20 cc. | — | alkaline. | 1 cc. | | 3.2 cc. |
| 5 | 0 | 13 cc. | 18.5 cc. | — | 1.5 cc. | 1 cc. | | 3 cc. |
| 6 | 0 | 13 cc. | 17.5 cc. | — | | trace. | | .6 cc. |
| 7 | 0 | 11 cc. | 22.5 cc. | — | alkaline. | 1 cc. | | .6 cc. |

The cultures of Table III were taken from the series of Table I at the time when they were at the maximum point of indol production and inoculated into a medium containing sugar, so as to observe the effect of sugar upon the proteolytic function.

Each culture was inoculated into alkali-peptone bouillon containing 0.5 per cent and 0.25 per cent of sugar respectively, and also into 25 cc. of the same bouillon, to which as much commercial lactic acid had been added as would require 10 cc. of a one-twentieth normal sodium hydroxide solution to neutralize it. After growth for five days at 37° to 38° C. the cultures were tested for acidity and for indol. The acidity is expressed in the number of cubic centimetres of a one-twentieth normal solution of sodium hydroxide that is required to neutralize 25 cc. of culture fluid. The results given in the table show that although these cultures were capable of producing an amount of indol in the alkali-peptone bouillon without sugar which required 10 to 13 cc. of a 0.1 per cent solution of indol to give the same color,

if 0.5 per cent of dextrose is added to the same medium it causes complete inhibition of indol production, and if 0.25 per cent is added only a slight reaction for indol is obtained. The culture containing lactic acid but no glucose produced a much greater amount of indol, and this fact shows that the acidity caused by fermentation is not the only cause of the cessation of the proteolytic function.

*Excess of the proteid function.*—The cultures mentioned above, that are tabulated in Tables I and II, and which were permitted to develop in a medium most favorable to proteolytic action and which was designed to exclude all other functions, will now be followed in their course for the purpose of determining the effect of an excess of this function.

The typical cultures of Table I, when carried through a series of cultures on a proteid medium, reach the height of indol production on the seventh day and in the second generation. Indol formation at that time is much greater than is found by the usual method of cultivation. After this period, however, indol production steadily decreases, and the culture becomes attenuated and dies in about the eighth generation. Like other attenuated cultures, if inoculated into dextrose bouillon the gas formation is less than normal.

In the cultures of Table II the reaction for indol does not appear until the fourth or fifth generation; it reaches its height in the following generation, then decreases and soon ceases. The attenuation of the cultures in these series is probably due to absence of fermentable carbohydrates, which are apparently necessary to make up the carbon deficit of the bacterial cells.

The indol curve of typical and atypical cultures is represented approximately in the chart on page 561.

We have made one experiment to determine whether cultures which are pathogenic when grown in the fluids of an animal form more indol under artificial cultivation than non-pathogenic cultures, thereby showing an increase of the proteolytic function. The virulence of the culture used was ascertained as soon as it was isolated from feces, and it was tested for indol. It was then kept for long periods on artificial media, with an occasional test for indol and virulence.

Finally it was again brought up to its normal condition by growth on a medium containing a large per cent of peptones.

As a result of this experiment we found that there was a parallelism between indol formation and pathogenesis in this instance. Conditions affecting the formation of indol affected the virulence also. The maximum amount of indol formed by this culture was not greater, however, than that formed by other cultures which were non-pathogenic. Of course this experiment does not prove that indol is formed in the peritoneal cavity. In a few tests made upon peritoneal exudate and the pus of abscesses which contained a pure culture of the bacillus coli communis, we have not obtained an indol reaction; but the cultures isolated from these fluids gave a genuine indol reaction in artificial media.

INDOL PRODUCTION CONSIDERED AS AN INDEX OF PATHOGENESIS.

A series of experiments relating to this question has been carried on for over a year on a virulent culture obtained October 6, 1895, at the autopsy of a case of parenchymatous nephritis. The fecal material was taken from the colon near the vermiform appendix. In this freshly isolated culture we noticed that gas production was about one-half the amount usually found in typical cultures of this organism derived from normal feces, but after keeping it upon agar-agar for two or three months the usual amount of gas was formed. Indol production was much greater than usual, and this fact led to a test of virulence. We found that 0.5 cc. of a bouillon culture about twenty-four hours old proved invariably fatal, by both subcutaneous and intraperitoneal inoculation, to guinea-pigs weighing 500 grammes, and 0.1 cc. of the same culture killed a guinea-pig weighing 280 grammes after several days, the organs of this animal being sterile at the time of its death.

This culture was then kept on agar-agar for about five months, when it was again tested on animals. It then required 1 to 2 cubic centimetres of a bouillon culture to kill an animal weighing from 300 to 500 grammes. A faint indol reaction was obtained on Dunham's solution. On March 21, 1896, it was placed in a freshly pre-

pared peptone solution, made by peptonizing casein with trypsin, and changed to a fresh culture tube on every third day. On March 28 indol formation had increased and guinea-pigs were again inoculated, a twenty-four-hour-old culture on the fresh peptone solution being used. The results were as follows:

| Date of inoculation. | Dose. | Weight. | Result. |
|---|---|---|---|
| March 28, | 0.3 cc. | 213 grammes. | Died in 18 hours. |
| " " | " | 216 " | " " 24 " |
| April 1, | 0.2 cc. | 213 " | " on 7th day. |
| March 30, | 0.3 cc.* | 190 " | Recovered. |
| April 4, | 0.5 cc.* | 195 " | Died in 18 hours. |
| " | 0.5 cc.* | 190 " | Recovered. |

The culture was again placed on ordinary agar-agar and remained with occasional changes until October 6, 1896 (a period of about six months). At that time there was an abundant growth on agar-agar, but no indol reaction could be obtained on either Dunham's or fresh casein-peptone solution. 3 cc. of a bouillon culture was required to kill an animal of 379 grammes weight. The culture was then changed to the peptonized casein solution and carried through a series of cultures. In the second generation of this series and the fifth day after its inoculation into the casein-peptone solution, a faint indol reaction was obtained, and on the tenth day the reaction was so intense that it required 15 cc. of a one-tenth indol solution to give the same degree of color to 25 cc. of the culture fluid. It was now inoculated, intraperitoneally, into animals with the following results:

| Date of inoculation. | Dose. | Weight. | Result. |
|---|---|---|---|
| October 27, 1896, | 1 cc. | 300 grammes. | Died in 24 hours. |
| " 30, " | 0.5 cc. | 340 " | " " 18 " |
| Nov. 19, " | 0.4 cc. | 355 " | " " " " |
| " " " | 0.3 cc. | 340 " | Recovered. |
| " " " | 0.2 cc. | 330 " | " |

Pure cultures of the organism inoculated were obtained in all the fatal cases from the spleen, liver, peritoneum and blood.

This culture, when regarded in the light of subsequent experiments upon the action of proteids on the functions of the colon bacillus, must

* 1% glucose bouillon was used in these experiments. See remarks above relating to fermentable carbohydrates. This difference in pathogenesis corresponds strikingly with lessening of the proteolytic function.

have been at the time of isolation under conditions which greatly favored the proteid function. This is shown by the excess of that function and the deficiency in gas production. Further cultivation on a medium containing both food-factors caused a balance of function, as seen by the increased gas production and decreased indol formation, without disappearance of either. Another culture of the colon bacillus that was isolated from the pus of an abdominal abscess, in which it was the only micro-organism present, presented the same deviation from the type and was caused to revert to the type by the same process.

The constant accentuation of indol production that we have observed to result from the continued cultivation of the organism in a medium especially adapted to this function is no greater in this instance than occurs in the majority of fresh cultures, many of which are either non-pathogenic or only slightly so. An estimation of the virulence of this organism by the amount of indol formed seems, for this reason, impracticable. Moreover, we have found that varieties of the colon bacillus which do not produce indol by the usual test may prove fatal to guinea-pigs in doses not exceeding 1 cc. of a bouillon culture. This fact would seem to indicate that although indol production as the final stage in the proteolytic function may be, like gas formation in fermentation, the first factor to be affected by changes unfavorable to this function, it does not prove that other important processes upon proteid material have not been carried on.

This experiment shows, however, that indol production and pathogenesis can both be increased by growth of a culture in a medium which contains an unusual amount of proteid material, so prepared as to be especially suitable for bacterial assimilation.

It has also been demonstrated above that the biological functions of the colon bacillus, including both indol formation and fermentation, may be materially modified by long-continued cultivation in media that are rich in proteid matters.

We have found by experiments, which we have given in detail, that the colon bacillus grown in an excess of proteid material in an artificial medium undergoes a disturbance of equilibrium in function, with first an increase of proteolytic activity and later an apparent suppression.

Cultures obtained from the pathological tissues of man and animals also present characteristics differing from those of the typical colon bacillus obtained from the healthy colon.

We turn now to the latter aspect of these phenomena and relate the results of experiments made for the purpose of ascertaining to what extent cultures taken from organs which have been undergoing inflammation for a considerable period of time differ in type from the normal colon and typhoid bacilli. For this test typhoid fever has seemed to afford exceptional advantages; for in this disease the products of inflammation are brought to the colon bacillus in the intestine, and later this organism often comes in contact with them in other viscera to which it finds access.

For the purpose of demonstrating this modifying effect upon cultures of bacteria belonging to the group under discussion, we obtained cultures directly from typhoid spleens and noted their reactions both upon carbohydrate and proteid foods. We have tried to classify these cultures according to these reactions, and with the following result: Cultures corresponding to the typical colon bacillus in reaction have not been found among the cultures studied. Of the forty-four cultures which were under observation, four cultures gave one or more of the prominent reactions of the colon bacillus and seemed to belong to the transitional series of the colon group. A second group gave the characteristic modifications in functions that are found in attenuated cultures of the colon group. A third and larger group gave reactions like those of the bacillus typhosus. These three groups, each differing considerably from the other in their reactions and each containing members which vary slightly, have been obtained from the spleens of typical cases of typhoid fever. May not the cultures of the first and second group be colon cultures which have been modified in function by growth in an excess of proteid food? The details of these experiments follow under series A, B, C, D.

TABLE IV.

GIVING THE RESULTS OF BACTERIOLOGICAL STUDIES UPON SPLEENS OBTAINED AT AUTOPSIES UPON CADAVERS OF PATIENTS DEAD OF TYPHOID FEVER. IN ALL CASES THE AUTOPSIES WERE MADE WITHIN 24 HOURS AFTER DEATH, AND THE SPLEENS WERE PLACED AT OUR DISPOSAL ALMOST IMMEDIATELY AFTERWARD. SERIES A.

| | Fermentation. | | | | | Indol formation during a series of changes in peptone solutions. | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Glucose. | | Lactose. | | | Changes in Dunham's solution. | | | | Changes in alkali-peptone bouillon. | | | |
| Series A. | Gas formation. | Acidity. | Gas formation. | Acidity. | Milk. | 1st gen. | 2nd gen. | 3rd gen. | 4th gen. | 1st gen. | 2nd gen. | 3rd gen. | 4th gen |
| $A^1$ | 0 | 2 cc. | 0 | 0 | coagulated and decolorized. | + | | | | 7 cc. | | | |
| $A^2$ | 0 | 2 cc. | 0 | 0 | very blue. | + | | | | + | | | |
| $A^3$ | 0 | 7 cc. | 0 | 0 | like control. | — | — | + | | — | + | | |
| $A^4$ | 0 | 10 cc. | 0 | 0 | like control. | — | — | — | + | — | — | — | + |
| $A^5$ | 0 | 11 cc. | 0 | 0 | like control. | — | — | — | — | — | — | — | — |
| $A^6$ | 0 | 11 cc. | 0 | 0 | like control. | — | — | — | — | — | + | | |
| $A^7$ | 0 | 10 cc. | 0 | 0 | like control. | — | — | + | | — | + | | |
| $A^8$ | 0 | 8.5 cc. | 0 | 0 | like control. | — | — | + | | — | + | | |
| $A^9$ | 0 | 8.5 cc. | 0 | 0 | coagulated and decolorized. | — | — | + | | — | — | + | |
| $A^{10}$ | 0 | 11 cc. | 0 | 0 | like control. | — | — | + | | — | — | + | |
| $A^{11}$ | 0 | 8.5 cc. | 0 | 0 | like control. | — | — | + | | — | — | + | |

Series A (Table IV). This series contains eleven cultures taken from single colonies having the appearance of typhoid colonies, on gelatine plates, inoculated with material from the spleen of a person who died in the Episcopal Hospital of Philadelphia, on the fifteenth day, of an attack of typhoid fever. The case was typical in every respect and the lesions observed at the autopsy were notably those of typhoid fever.*

*Fermentation.*—These cultures in one per cent dextrose and lactose bouillon showed absence of gas production. The amount of lactic acid formed by each culture was estimated by the phenolphthalein method,

* We wish here to express our obligations to Dr. H. W. Cattell, Coroner's Physician, Philadelphia, through whose courtesy the pathological material used in these studies was placed at our disposal.

namely, by adding one cubic centimetre of a solution of phenolphthalein (2.5 grammes to 500 cc. of 50 per cent alcohol). This was brought to as sharp a neutralization as possible by titrating with a one-twentieth normal solution of sodium hydroxide. The amount required to neutralize the culture fluid was estimated from this result. In every instance where the acidity is designated it is represented by the number of cubic centimetres of a one-twentieth normal solution of sodium hydroxide that would be required to neutralize 25 cc. of the culture fluid.

The amount of acid formed by the members of this series, which is designated as series A, in glucose bouillon, with the exception of $A^1$ and $A^2$, corresponds to that produced by cultures of the bacillus typhosus, as will be seen by comparing the table containing this series (Table IV) with that of Table VIII. $A^1$ and $A^2$ formed very little acid and did not grow in the closed arm of the fermentation tube. In lactose bouillon no acid was produced by this series.

These cultures behaved in litmus milk as follows: $A^1$ coagulated and decolorized milk in twenty-four hours, and $A^9$ in twenty-five days. $A^2$ turned milk intensely blue. The remainder of the series gave the usual reaction seen in typhoid cultures in milk.

*Indol production.*—These cultures were inoculated into each of the peptone solutions, the alkali-peptone bouillon and Dunham's peptone solution, and incubated for three days, when they were tested for indol. $A^1$ gave a distinct indol reaction; the remainder of the series was negative as regards the reaction in both of the peptone solutions. By continued culture in both of the peptone solutions, however, every member of the series except $A^5$ was, after varying periods of time, *brought to indol production*, so that a decided reaction was obtained.

Series B (Table V). These cultures were obtained from the spleen of a person who died of a relapse of typhoid fever. The spleen was characteristic of the disease, and the intestine showed ulceration of Peyer's patches. Of the cultures tabulated, $B^1$ to $B^9$ were taken from colonies on gelatine plates, having the appearance of typhoid colonies; $B^{10}$ to $B^{15}$ were taken from large, yellowish, surface colonies on Elsner's medium which were supposed to be the bacillus coli communis.

*Fermentation.*—In this series, also, there was no gas production in media containing either dextrose or lactose, and the amount of acid formed in dextrose bouillon was about the same as in series A, and in lactose bouillon was entirely wanting. $B^7$ differed from the others by forming a much smaller amount of acid in dextrose bouillon and by coagulating milk after 16 days; $B^{12}$ coagulated milk in 17 days. The remainder of the series gave, in milk, the reaction given by the typhoid bacillus.

TABLE V.

GIVING THE RESULTS OF BACTERIOLOGICAL STUDIES UPON SPLEENS OBTAINED AT AUTOPSIES UPON CADAVERS OF PATIENTS DEAD OF TYPHOID FEVER. SERIES B.

| Series B. | Fermentation. | | | | | Indol formation during a series of changes in peptone solutions. | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Glucose. | | Lactose. | | | Changes in Dunham's solution. | | | Changes in alkali-peptone bouillon. | | |
| | Gas formation. | Acidity. | Gas formation. | Acidity. | Litmus milk. | 1st gen. | 2nd gen. | 3rd gen. | 1st gen. | 2nd gen. | 3rd gen. |
| $B^1$ | 0 | 7.5 cc. | 0 | 0 | like control. | — | — | — | — | + | |
| $B^2$ | 0 | 8 cc. | 0 | 0 | like control. | — | — | — | — | + | |
| $B^3$ | 0 | 7 cc. | 0 | 0 | like control. | — | — | — | — | — | — |
| $B^4$ | 0 | 7.5 cc. | 0 | 0 | like control. | — | + | | — | + | |
| $B^5$ | 0 | 7.5 cc. | 0 | 0 | like control. | — | + | | — | + | |
| $B^6$ | 0 | 6.5 cc. | 0 | 0 | like control. | — | + | | — | + | |
| $B^7$ | 0 | 2 cc. | 0 | 0 | coagulated milk in 16 days. | — | + | | — | — | + |
| $B^8$ | 0 | 7.5 cc. | 0 | 0 | like control. | — | — | + | — | + | |
| $B^9$ | 0 | 7.5 cc. | 0 | 0 | like control. | — | + | | — | + | |
| $B^{10}$ | 0 | 12 cc. | 0 | 0 | very blue. | — | + | | + | | |
| $B^{11}$ | 0 | 9 cc. | 0 | 0 | very blue. | — | + | | + | | |
| $B^{12}$ | 0 | 4.5 cc. | 0 | 0 | coagulated in 17 days. | — | + | | + | | |
| $B^{13}$ | 0 | 8 cc. | 0 | 0 | like control. | — | + | | + | | |
| $B^{14}$ | 0 | 6.5 cc. | 0 | 0 | like control. | — | + | | + | | |
| $B^{15}$ | 0 | 6.5 cc. | 0 | 0 | like control. | + | | | + | | |

*Indol formation.*—All of these cultures formed indol at periods which are indicated in Table V. $B^{10}$ to $B^{15}$ gave somewhat deeper reactions than $B^1$ to $B^9$.

Series C (Table VI). The spleen used for this series was obtained at the autopsy of a person who died in the Presbyterian Hospital of Philadelphia, on the twenty-first day, of an attack of typhoid fever.

*Fermentation.*—In the eleven cultures which form this series there was absence of gas formation with both dextrose and lactose bouillon in all except $C^1$, which gave with dextrose an amount equal to one-fourth

of the arm of the fermentation tube.* The acidity of several of these cultures in dextrose bouillon was somewhat greater than has been found by us in typhoid cultures, equaling that of many cultures of the bacillus coli communis. In milk, cultures 3, 5 and 7 caused coagulation, and the remainder gave reactions similar to the typhoid bacillus.

TABLE VI.

GIVING THE RESULTS OF BACTERIOLOGICAL STUDIES UPON SPLEENS OBTAINED AT AUTOPSIES UPON CADAVERS OF PATIENTS DEAD OF TYPHOID FEVER. SERIES C.

| Series C. | Fermentation. | | | | | Indol formation during a series of changes on peptone solutions. | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Glucose. | | Lactose. | | | Changes in Dunham's solution. | | | Changes in alkali-peptone bouillon. | | |
| | Gas formation. | Acidity. | Gas formation. | Acidity. | Litmus Milk. | 1st gen. | 2nd gen. | 3rd gen. | 1st gen. | 2nd gen. | 3rd gen. |
| $C^1$ | 0 | 15 cc. | 0 | 0 | like control. | + | | | 10 cc. | | |
| $C^2$ | 0 | 3.5 cc. | 0 | 0 | like control. | + | | | 7.5 cc. | | |
| $C^3$ | 0 | | 0 | 0 | coagulated in 15 days. | + | | | 10.5 cc. | | |
| $C^4$ | 0 | 10 cc. | 0 | 0 | like control. | + | | | 10 cc. | | |
| $C^5$ | 0 | 15 cc. | 0 | 0 | coagulated in 15 days. | — | | Reaction on 16th day. | — | | Reaction on 16th day. |
| $C^6$ | 0 | | 0 | 0 | like control. | + | | | 7.5 cc. | | |
| $C^7$ | ¼ of closed arm of tube. | 5 cc. | 0 | 0 | coagulated in 24 hours. | + | | | 10 cc. | | |
| $C^8$ | 0 | 17.5 cc. | 0 | 0 | like control. | + | | | 10 cc. | | |
| $C^9$ | 0 | 11.5 cc. | 0 | 0 | like control. | — | | + | — | — | + |
| $C^{10}$ | 0 | 12.5 cc. | 0 | 0 | like control. | — | | + | — | — | + |
| $C^{11}$ | 0 | | 0 | 0 | | + | | | + | | |

*Indol production.*—This series differed greatly from A and B by forming a much greater amount of indol. After growing in the alkali-peptone bouillon for three days, all except $C^5$, $C^9$ and $C^{10}$ gave a reaction equal to that of the typical colon bacillus under the same conditions. These three cultures, after growth in peptone solutions for several days, produced enough indol to give a decided reaction.

* The amount of gas formed by the typical bacillus coli communis in one per cent dextrose bouillon is sufficient to fill one-half of the closed arm of the fermentation tube.

TABLE VII.

GIVING THE RESULTS OF BACTERIOLOGICAL STUDIES UPON SPLEENS OBTAINED AT AUTOPSIES UPON CADAVERS OF PATIENTS DEAD OF TYPHOID FEVER. SERIES D.

| Series D. | Fermentation. | | | | | Indol formation during a series of changes in peptone solutions. | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Glucose. | | Lactose. | | | Changes in Dunham's solution. | | | Changes in alkali-peptone bouillon. | | |
| | Gas formation. | Acidity. | Gas formation. | Acidity. | Litmus milk. | 1st gen. | | | 1st gen. | | |
| $D^1$ | $\frac{2}{3}$ of closed arm of tube. | 7.5 cc. | Bubble. | ? | very blue. | + | | | + | | |
| $D^2$ | 0 | 2.5 cc. | 0 | 0 | coagulated in 10 days. | + | | | + | | |
| $D^3$ | 0 | .5 cc. | 0 | 0 | very blue. | + | | | + | | |
| $D^4$ | 0 | 2.5 cc. | 0 | 0 | decolorized but not coagulated. | + | | | + | | |
| $D^5$ | 0 | 2 cc. | 0 | 0 | lighter than control. | + | | | + | | |
| $D^6$ | 0 | 10 cc. | 0 | 0 | very blue. | + | | | + | | |
| $D^7$ | 0 | 3 cc. | 0 | 0 | very blue. | − | | | + | | |
| $D^8$ | 0 | 2 cc. | 0 | 0 | about like control. | − | | | + | | |
| $D^9$ | 0 | 3.5 cc. | 0 | 0 | very blue. | + | | | + | | |

Series D (Table VII). The spleen used for these cultures was also obtained at the autopsy of a case of typhoid fever. The mucous membrane of the intestine was eroded in large areas, Peyer's patches were ulcerated and perforation had taken place. Gelatine plates inoculated with the splenic tissue were much overgrown with liquefying organisms which, from subsequent culture, proved to be a species of the bacillus subtilis. The cultures selected for this series gave, on gelatine, the characteristic appearance of the colon group.

*Fermentation.*—$D^1$ gave a small amount of gas with both dextrose and lactose; the others were not gas-forming. The greater number of these cultures showed very little growth in the closed arm of the fermentation tube, and a much smaller quantity of acid was estimated than in the previous series. In milk, $D^2$ caused coagulation and decolorization; the remaining members of the series varied slightly or not at all from the control tube at first, and then turned the milk very blue.

*Indol production.*—All of these cultures except $D^7$ and $D^8$ gave a decided indol reaction after growth for three days in Dunham's solution, and these cultures gave the reaction in the same period when grown in alkali-peptone bouillon.

In studying the four series of cultures obtained from typhoid spleens we have been not a little puzzled to determine whether we are dealing with the typhoid bacillus or with other members of the colon group. Many of these cultures cannot be differentiated from the typhoid organism by any of the tests usually employed. They conform to its reactions regarding fermentation, that is, in gas formation, acidity, absence of inverting enzyme, and growth in milk. The indol reaction is negative *when tested by the usual method.* Are the individual cultures of this series which do thus conform to the characteristics of a typhoid culture the true typhoid bacillus, or are they members of the colon group which give similar reactions and are known as pseudo-forms of the typhoid bacillus? Their source, the spleens of typical cases of typhoid fever, would indicate that they are typhoid cultures, but the colon bacillus, as has been shown by Welch (14), has also frequent opportunity, in typhoid fever, for passing from the intestine to other viscera, and is often found in them, though, strange to say, there are no trustworthy statistics as to the frequency of the occurrence of the colon bacillus in the spleen in cases of genuine typhoid fever.

Among the cultures forming these series we have been able to distinguish groups having slightly different characteristics; for instance, $A^1$, $A^9$, $C^3$, $C^5$ and $C^7$ break up lactose, as shown by the coagulation of milk. $A^1$ and $D^1$ give an indol reaction by the usual test. $C^7$ and $D^1$ form a small amount of gas in dextrose bouillon. From this may we not reasonably regard these cultures as representing colon bacilli that have been modified in their characteristic biological functions through exposure to the vital resistance of tissues with which they are not naturally concerned; or are we, in the light of our culture tests with excessive amounts of proteid material, to consider them as modified by their development in nutritive material excessively rich in proteids; or, still again, is this assumed attenuation due to the detrimental influence of the inflammatory products, particularly the leucocytes or their protective secretions that have recently attracted so much attention?

The cultures of the D series, except $D^1$, which has been included in

the group just mentioned, are marked by the small amount of acid formed in dextrose bouillon, by the intensely blue color of litmus milk, and the reaction for indol by the usual test. $A^2$ and $A^7$ are like this series in reaction. These cultures may be considered as forming a separate group.

The remaining cultures of these series conform to the reactions of the typhoid bacillus in every respect when the usual tests are employed, *but they form indol in varying amounts when cultivated in a medium which is unusually favorable for its formation.*

An attempt has been made to classify these groups by comparing them, first with attenuated cultures, then with transitional forms, and finally with typhoid cultures.

A series of colon cultures that have become attenuated by long residence in feces show the following characteristics: There is an almost entire loss of the function of fermentation, shown by absence of gas formation, by the very small amount of acid formed in dextrose bouillon, and the intensification of the blue color of litmus milk. A microscopic examination shows small bacilli that are difficult to distinguish from cocci.

By a comparison of our three groups with the cultures of attenuated colon bacilli we find that our second group, which includes $A^2$, $B^7$, and all of the D series except $D^1$, corresponds in reactions to the attenuated cultures, and, therefore, it seems not unlikely that they are such cultures.

The lesions of the intestine in the case from which the D series was obtained, namely, the almost complete erosion of the mucous membrane and the perforation and peritonitis, seem to indicate that the products of inflammation had afforded large amounts of proteid material for a period of time sufficient to cause an attenuation of the colon bacillus by an excess of a single food factor. We do not, of course, insist upon this interpretation, but the peculiar attenuation to which reference has been made, in regard to cultures grown in artificial media very rich in proteids, *i. e.* the fresh alkali-peptone bouillon, inclines us to this view.

Our third and most important group varies from the attenuated

cultures in the following points: They form large quantities of acid in glucose bouillon and do not show a markedly alkaline reaction in litmus milk. The indol reaction is negative in the peptone solution of Dunham, unless the cultures are frequently transplanted into a fresh medium. Under the microscope these bacteria resemble ordinary cultures of the typhoid or colon bacillus. The cultures of our third group, therefore, show biological traits varying from attenuated cultures, and also from the so-called transitional forms described by Smith, Stoecklin and others, which are changing in type and approach either one or the other of the extreme varieties of this group.

Among the cultures comprising these series we find two, $C^{7}$ and $D^{1}$, which approach quite closely to the colon bacillus in type. They form a small amount of gas in glucose bouillon and give a marked indol reaction by the usual test. $C^{7}$ coagulates milk in twenty-four hours. Four other cultures—$A^{1}$, $A^{9}$, $C^{3}$ and $C^{5}$—also coagulate milk. Can we not reasonably regard these cultures as in a transitional state?

A considerable number of varieties comprised in the colon group have been described by various writers, and because of their striking likeness to the typhoid bacillus they have been designated as pseudo-typhoid bacilli. As a further means, therefore, in classifying these cultures and thereby to distinguish them from or identify them with the typhoid bacillus, we have employed two additional tests.

Our first test has been made with a number of typhoid cultures that we have obtained through the courtesy of several of the best-known laboratories in the country.* These cultures have been grown in alkali-peptone bouillon and Dunham's solution to find whether, by a series of transplantations in these media, they may not also be brought to indol formation; for if this reaction be obtained, then our third group corresponds to the typhoid bacillus in all cultural peculiarities. That indol formation might really occur in typhoid cultures was first suggested to us in 1895, when we were testing cultures obtained from a typhoid spleen, and which gave all the differential reactions of typical typhoid cultures. Repeated tests were made for

* We desire here to express our obligation to the gentlemen of these laboratories, who have so obligingly placed their cultures at our disposal.

indol, because in some of the cultures a slightly pinkish tint indicated the indol reaction, but it was finally supposed to be due to indol contained in the peptone which formed a constituent of the solution.

Péré did not obtain the indol reaction in typhoid cultures which had grown for many days in his casein-peptone solution. While our tests were being made, however, an indol-forming typhoid culture has been sent to us by Dr. Flexner, from the Pathological Laboratory of the Johns Hopkins University.

*Typhoid cultures tested for indol.*—The cultures of this series, seventeen in number, were obtained by us from the different laboratories mentioned in Table IX at the end of this article. All of the usual tests for the differentiation of the typhoid organism have been made, and the results obtained are given in detail in Table VIII.

In dextrose bouillon all of these cultures formed moderately large amounts of lactic acid without gas production. In lactose bouillon and in milk no perceptible reaction was obtained. On potato the cultures showed the usual moist, almost invisible growth.* Tests for indol in a one per cent dried-peptone solution gave a negative result. Microscopically these cultures gave the usual non-characteristic morphology of the typhoid bacillus.

After ascertaining that these cultures gave the commonly recognized characteristic reactions of the typhoid bacillus, they were tested upon the two kinds of peptone solutions that were employed in making the tests upon the colon bacillus. A transplantation into fresh medium was made every seventy-two hours and the old cultures were first examined microscopically and then tested for indol by the method heretofore described.

This series of tests is noted in Table VIII. *The indol reaction was obtained in every culture, and the greater number were brought to indol production by the termination of the third period of cultivation.* Control flasks of sterile media were also tested for indol and gave a negative result. These cultures, like the atypical series of the colon group, did not produce large amounts of indol, but gave, nevertheless, an unmistakable reaction.

* To eliminate the error arising from the use of different potatoes, all of the tubes for this test were prepared from one large potato, which gave a decidedly acid reaction.

TABLE VIII.

GIVING THE RESULTS OF THE STUDY OF CULTURES OF THE BACILLUS TYPHOSUS, OBTAINED FROM DIFFERENT LABORATORIES, WITH SPECIAL REFERENCE TO FERMENTATION AND INDOL FORMATION.

| No. of culture. | Fermentation. | | | | | | Indol formation during a series of changes in peptone solutions. | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Glucose. | | Lactose. | | | Potato. | Changes in Dunham's solution. | | | | Changes in alkali-peptone bouillon. | | | |
| | Gas formation. | Acidity. | Gas formation. | Acidity. | Milk. | No distinct growth. | 1st gen. | 2nd gen. | 3rd gen. | 4th gen. | 1st gen. | 2nd gen. | 3rd gen. | 4th gen. |
| 1 | 0 | 6.5 cc. | 0 | 0 | slightly pinker than control. | no distinct growth. | — | — | + | | — | + | | |
| 2 | 0 | 8.5 cc. | 0 | 0 | slightly pinker than control. | no distinct growth. | — | — | — | — | — | — | — | + |
| 3 | 0 | 10.5 cc. | 0 | 0 | slightly pinker than control. | no distinct growth. | — | — | + | | — | + | | |
| 4 | 0 | 11 cc. | 0 | 0 | slightly pinker than control. | no distinct growth. | + | | | | + | | | |
| 5 | 0 | 9 cc. | 0 | 0 | slightly pinker than control. | no distinct growth. | — | — | + | | — | + | | |
| 6 | 0 | 9.5 cc. | 0 | 0 | slightly pinker than control. | no distinct growth. | — | — | + | | — | + | | |
| 7 | 0 | 10 cc. | 0 | 0 | slightly pinker than control. | no distinct growth. | — | — | + | | — | — | + | |
| 8 | 0 | 10 cc. | 0 | 0 | slightly pinker than control. | no distinct growth. | — | — | + | | — | + | | |
| 9 | 0 | 11 cc. | 0 | 0 | slightly pinker than control. | no distinct growth. | — | — | + | | — | — | + | |
| 10 | 0 | 7.5 cc. | 0 | 0 | slightly pinker than control. | no distinct growth. | — | — | + | | — | — | + | |
| 11 | 0 | 3.5 cc. | 0 | 0 | slightly pinker than control. | no distinct growth. | — | — | + | | — | — | — | + |
| 12 | 0 | 9 cc. | 0 | 0 | slightly pinkish color. | no distinct growth. | — | — | + | | — | + | | |
| 13 | 0 | 8.5 cc. | 0 | 0 | slightly pinkish color. | no decided growth. | — | — | — | | — | — | + | |
| 14 | 0 | 9.5 cc. | 0 | 0 | slightly pinkish color. | no decided growth. | — | — | + | | — | + | | |
| 15 | 0 | | 0 | 0 | slightly pinkish color. | no decided growth. | — | — | + | | — | — | + | |
| 16 | 0 | | 0 | 0 | slightly pinkish color. | no decided growth. | — | — | + | | — | — | + | |
| 17 | 0 | | 0 | 0 | slightly pinkish color. | no decided growth. | — | — | + | | — | + | | |

In comparing this series with those obtained by us directly from typhoid spleens we find that series A and B from spleens gave amounts of indol corresponding to those formed by the typhoid cultures obtained by us from different laboratories when tested under the same conditions, while all of series C, except three cultures, gave a much larger amount.

The results obtained in the foregoing experiments demonstrate several interesting and important points, viz., that the bacillus typhi abdominalis assumes the indol-producing function when cultivated under special conditions; that the colon bacillus may lose its indol-producing function when grown under conditions that at first favor the exercise of this function, but which finally cause it to lose the property; and last, that the conditions favoring an increase of indol production in the colon bacillus, and an assumption of this property by an organism usually devoid of it, viz. the typhoid bacillus, are the same in both instances.

Our final test for the differentiation of our cultures obtained from typhoid spleens is the serum test, to which the investigations of Widal have recently drawn so much attention that it has become a well-known and popular diagnostic procedure in typhoid fever. We have reversed this test by using it for testing our cultures which correspond in other reactions to the typhoid bacillus. For the sake of constancy, all of our cultures, both typhoid and colon, were first tested with a single specimen of blood that was obtained from a genuine case of typhoid fever, and which had given a characteristic reaction with the typhoid culture used by us for the diagnosis of typhoid fever by the so-called method of Widal. They were then subjected to a second test with blood from another case of the disease that likewise had given a typical reaction with our test culture. The results of both tests were identical. They are tabulated in Table IX, which will be found at the end of this article, pp. 587–591.

We have used for our tests bouillon cultures which were about eighteen hours old, from which we have prepared a hanging drop by mixing a loopful of the culture with a loopful of diluted dried blood.

The hanging-drop preparation was then observed for one or more

hours, noting the occurrence of clumping and the cessation of motion, both around the borders of the masses and in the single bacilli which are seen in the deeper portions of the drop. These points have been tabulated, so that a comparison may be made of the reactions given by typhoid cultures obtained from different sources, and also of the reactions of other members of the colon group.

We have ascribed a positive reaction to cultures in which there has been an almost complete agglutination of the bacilli into masses, with cessation of motility in the masses and also in the greater number of the few remaining solitary bacilli. In every specimen of blood that we have tested with a reliable typhoid culture, and where a characteristic reaction in every other respect has been obtained, a few actively motile bacilli, with an easily detected translatory movement, have been seen in the depths of the hanging drop, notwithstanding the fact that the preparation may have been under observation for several hours. We have recorded such a reaction as positive, although we are aware that Johnston and McTaggart (15) consider that only those reactions are typical in which there is complete clumping and total arrest of motion. These reactions they obtain in a young attenuated culture, but we have not been able to obtain it in fresh cultures of a specimen of the typhoid bacillus that has been under artificial cultivation for about nine years, its original source having been the Hygienic Institute at Berlin.

By referring to the table it will be seen that only about one-third of our typhoid cultures gave what is regarded by us as a positive reaction, although every culture except No. 17 showed a distinct agglutination of the bacilli and some degree of cessation of motility. It is proper to state in connection with culture No. 17, however, that it gave certain cultural reactions not common to the typical typhoid bacillus, notably a striking intensification of the blue color of litmus milk.

A comparison of the reactions given by our typhoid cultures with those of our cultures obtained from spleens shows that every member of the A series from spleens gave as typical a reaction as any one of the typhoid cultures. Of this series, $A^1$ and $A^9$ were classified as transitional colon cultures because they ferment lactose. The remaining cultures of this series were placed in the typhoid bacillus group.

Of the B series from spleens about one-half of the cultures gave a positive serum reaction. The remaining cultures all showed immediate clumping in large masses, but varied much in regard to the cessation of motility, some showing but little evidence of loss of motion. $B^{10}$ and $B^{11}$ gave rapid agglutination of the bacilli with marked cessation of motion, but became motile again at the end of an hour, so that the masses became somewhat disentangled. These cultures correspond in reaction to Nos. 2 and 15 of our typhoid cultures.

Of five cultures of the C series from spleens which were tested with the typhoid blood, four gave a positive reaction. The cultures of the D series from spleens did not give any indications of a reaction.

We find, therefore, that of 28 cultures derived from typhoid spleens and giving all of the cultural reactions of typhoid bacilli, 18 gave a positive reaction, while 10 did not fulfill the requirements of this reaction in regard to cessation of motion. Two cultures classified as transitional colon cultures gave as typical a reaction as any of the cultures tested.

Our tests upon the typical and atypical series of the bacillus coli communis show that in one culture of each series, No. 8 of the typical and No. 1 of the atypical, a reaction was obtained which seemed to us as striking as any reaction obtained by us with typhoid cultures. Several of the other cultures differed from the control drop by presenting indications of a reaction, as will be seen by the table in which the prominent features of each reaction are noted.

Our observations upon these cultures, about seventy in number, seem to us to show that there are difficulties attending the diagnosis of typhoid cultures by this method. Negative reactions have been obtained in many cultures which we regard as typhoid organisms, and a positive reaction has been noted in two different members of the species bacillus coli communis. Between these extremes in reaction are those which show one prominent feature with partial absence of the other, and many other variations which are not found in the control culture drop.

In view of the fact that nearly all observers agree in the statement that the reaction between genuine typhoid serum and typhoid bacilli

is not the same for all specimens of the organism, but occurs in a striking way with cultures possessing certain peculiarities, and fails entirely with other cultures devoid of these characteristics, though the grounds for regarding them as typhoid bacilli may be equally strong in both instances, we are not inclined to consider this test in the method of its application employed in these investigations as entirely trustworthy for the differentiation of allied bacteria.

It is needless to open the discussion at this point regarding the influence of various dilutions of serum upon the result of the test. It will suffice to say that in the method employed, notably that of dissolving the agglutinating substance from dried blood by a drop of water, it is impossible to obtain either accurate or constant degrees of dilution, so that we may consider certain of our irregularities to be due in part to this defect of the method.

## CONCLUSIONS.

Assuming the typical colon bacillus and the typical typhoid bacillus to represent the types of this group that present the greatest divergences in biological peculiarities, we conclude, as others also have done, that there is a series of closely related forms that may be regarded as intermediate or transitional and which serve to establish a biological relationship, either near or remote, between these two typical members.

From our own studies we are inclined to regard the typical colon bacillus as the type of this group, for the reason that its functional equilibrium, as observed in the intestine, is so permanent a quality that it may readily be perpetuated under what is ordinarily regarded as favorable artificial circumstances, and that with the continuance of such conditions there is no conspicuous tendency on the part of this organism to deviate from what we regard as its norm; whereas, on the other hand, with all the other members of this group with which we have worked, there is not only a lack of uniformity in the adjustment of the functions, but such as exists is readily disturbed under artificial environment; though it must be borne in mind that even with the typical colon bacillus we have also shown functional modifications to be possible under particular conditions.

When the members of the colon group are cultivated under circumstances favorable to the development of both the function of fermentation and that of proteolysis, fermentation invariably takes precedence and no evidence of proteolysis is manifested until after fermentation has ceased.

The cultivation of all the members of the colon group under circumstances that favor the development of one function, viz. that of proteolysis, at the expense of another, viz. that of fermentation, results first in an apparent increase of vigor; but this is of temporary duration and is quickly followed by the decline and death of the cell.

The result of this increased activity of the proteolytic function is the formation of much larger amounts of indol by typical colon cultures than has ever been obtained by us by any other method.

By the method of experimentation through which we were enabled to accentuate the proteolytic activity of the typical colon bacillus, as caused by an increase of indol formation, we have also induced the function of indol formation not only in atypical colon bacilli that had been devoid of it, but in every specimen of typical typhoid bacilli to which we had access as well.

We feel justified in regarding one of the differential tests between the typhoid and colon bacillus, namely that of indol formation on the part of the latter and the absence of this function from the former, as of questionable value, for the reason, as shown above, that by particular methods of cultivation indol production has been shown to accompany the development of a number of specimens that we have every reason to regard as genuine typhoid bacilli.

As a result of our own experiments, together with the observations of others, there can be no doubt that the bacillus coli communis at times possesses pathogenic properties, and that by artificial methods of treatment it may often be brought from a condition of benignity to one of virulence.

The spleen of a typhoid patient has always been regarded as the only trustworthy source from which to obtain the typical typhoid bacillus. While we believe this to be true, still our investigations show that other members of the colon group may also be present in

this viscus; in fact, from such spleens we have isolated practically all of the varieties of this group with which we are acquainted.

From our experience, the value of the serum test for the differentiation of typhoid and colon bacilli would seem to be questionable. We are inclined, however, to attribute the irregularities recorded above as due more to the method of application than to defects of the principles involved; for, as stated, by the use of dried blood, as in our experiments, it is not possible to make the test with constant and accurate, or even approximately accurate, dilutions of the serum. Our irregularities may be in part due to this defect. We therefore lay less stress upon this than upon the other features of our work.

In closing, we desire to thank Dr. A. C. Abbott, the Director of the Laboratory of Hygiene, for many valuable suggestions in conducting this work.

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TABLE IX.

SHOWING RESULTS OF TESTS WITH TYPHOID BLOOD UPON TYPHOID AND COLON BACILLI FROM VARIOUS SOURCES.

| Culture. | Source of culture. | Results of test with typhoid blood by the dry method of Johnston. | Remarks. |
|---|---|---|---|
| No. 1 | Pathological Laboratory, McGill University. | + | Almost immediate complete clumping, entire cessation of motion in the clumps and in the single bacilli, except a few actively motile ones in the depths of the hanging drop. |
| No. 2 | Department of Pathology, College of Physicians and Surgeons, New York City. | ± | Immediate clumping of nearly all of the bacilli, with cessation of motion; at the end of two hours the masses are divided and there is active motion throughout the field. |
| No. 3 | Johns Hopkins University. Labeled (Gall-bladder). | ± | Very slow, but finally characteristic clumping. The active motion seen at first in the masses finally almost ceased, but the single bacilli remained actively motile. |
| No. 4 | Johns Hopkins University. Designated (B). | + | Almost instantaneous agglutination in large masses. Entire cessation of motion in the clumps; an occasional motile bacillus. |
| No. 5 | Laboratory of Bacteriology, Board of Health of the Province of Quebec. Designated (A). | — | Darting movement of the bacilli, slowly forming and small clumps with decided motion around the borders. Single bacilli are motile. |
| No. 6 | Do. Designated (B). | — | Immediate and characteristic clumping; motility much lessened, but did not cease. |

TABLE IX.—*Continued.*

| Culture. | Source of culture. | Results of test with typhoid blood by the dry method of Johnston. | Remarks. |
|---|---|---|---|
| No. 7 | Laboratory of Bacteriology, Board of Health of the Province of Quebec. Designated (C). | — | Slow and incomplete agglutination, very little diminution of motility. |
| No. 8 | Do. Designated (D). | — | Slow and incomplete clumping. Active motion around the borders of the masses and of the single bacilli. |
| No. 9 | Do. Designated (E). | — | Immediate clumping of small masses, and later of large ones. Considerable motion throughout the field. |
| No. 10 | University of Michigan. Labeled Typhoid (Jord.) | — | Darting movement, immediate formation of clumps which retain a swaying motion. Single bacilli actively motile. |
| No. 11 | University of Michigan. Labeled Eberth. | + | Characteristic clumping. Cessation of motion except in a few solitary bacilli. |
| No. 12 | Laboratory of Pathology, Mass. General Hospital. Designated (20). | — | Slow clumping of bacilli, swaying movement of the masses. Single bacilli actively motile. |
| No. 13 | Laboratory of Pathology of Mass. General Hospital. Designated (22). | ± | Formation of clumps and considerable diminution of motion, but not complete cessation in the clumps or in single bacilli. |
| No. 14 | Bacteriological Laboratory, Health Department, New York City. | + | Immediate clumping, cessation of motion in the clumps and in all except a few of the single bacilli. |
| No. 15 | Laboratory of Hygiene, Univers. of Penn. Designated D 2. | + | Immediate clumping, cessation of motion in the clumps and in all except a few of the single bacilli. |
| No. 16 | Laboratory of Hygiene, Univers. of Penn. Designated E 1. | ± | Immediate agglutination into large clumps, with cessation of motion. After two hours, separation of the bacilli in the masses and active motion of the single bacilli. |
| No. 17 | Johns Hopkins University (1895). Designated F′. | — | Appearance very similar to that of the control drop. |
| $A^1$ | Typhoid Spleen. | + | Immediate clumping, with struggling motion; later complete cessation of motion in the clumps and in the single bacilli on the surface of the hanging drop. Bacilli in the depths of the drop are motile. |
| $A^3$ | " | + | Slow adhesion of bacilli which spin rapidly on the vertical axis. Clumps cease motion, but a few single bacilli in the depths of drop retain motility. |
| $A^4$ | " | + | Immediate clumping, with struggling movement in the clumps which soon ceased. A few single bacilli remain actively motile. |

TABLE IX.—*Continued.*

| Culture. | Source of culture. | Results of test with typhoid blood by the dry method of Johnston. | Remarks. |
|---|---|---|---|
| $A^5$ | Typhoid Spleen. | + | Immediate clumping, cessation of motion except in an occasional bacillus. |
| $A^6$ | " | + | Immediate clumping, cessation of motion except in a few single bacilli. |
| $A^7$ | " | + | Immediate clumping, cessation of motion except in a few single bacilli. |
| $A^8$ | " | + | Masses gather slowly, but cessation of motion except in a few single bacilli. |
| $A^9$ | " | + | Rapid clumping, cessation of motion except in a few single bacilli. |
| $A^{10}$ | " | + | Rapid clumping, cessation of motion except in a few single bacilli. |
| $A^{11}$ | " | + | Rapid clumping, cessation of motion except in a few solitary bacilli. |
| $B^1$ | " | + | Rapid agglutination, cessation of motility except in an occasional bacillus. |
| $B^2$ | " | — | Rapid clumping, but both the clumps and the single bacilli retain motility. |
| $B^3$ | " | — | Rapid clumping, but both the clumps and the single bacilli remain motile. |
| $B^4$ | " | + | Clumping in large motionless masses. A few single bacilli in the deep portions of the drop remain motile. |
| $B^5$ | " | + | Clumping in large motionless masses. A few solitary bacilli remain motile. |
| $B^6$ | " | — | Agglutination takes place very slowly, many single actively motile bacilli. |
| $B^8$ | " | + | Rapid clumping, cessation of motility except in a few single bacilli. |
| $B^9$ | " | — | Clumping takes place slowly, many single motile bacilli. |
| $B^{10}$ | " | ± | Immediate clumping, with marked cessation of motility. An hour later the masses show a struggling movement and single bacilli are motile. |
| $B^{11}$ | " | — | Agglutination of a sheet of bacilli on the surface of the drop adjoining the cover-slip; later broken into clumps with struggling motion. |
| $B^{12}$ | " | — | Rapid clumping, with but little cessation of motility at the end of two hours. |
| $B^{13}$ | " | — | Rapid clumping, but little cessation of motion around borders of clumps or in the single bacilli at the end of two hours. |
| $B^{14}$ | " | — | Rapid clumping, movement over the entire field at the end of two hours. |
| $B^{15}$ | " | + | Rapid clumping, cessation of motion except in a few single bacilli. |
| $C^3$ | " | + | Rapid and almost complete clumping of the bacilli, with cessation of motion except in a few single bacilli. |

TABLE IX.—*Continued.*

| Culture. | Source of culture. | Results of test with typhoid blood by the dry method of Johnston. | Remarks. |
|---|---|---|---|
| $C^5$ | Typhoid Spleen. | + | Rapid clumping and complete absence of translatory motion. |
| $C^6$ | " | + | Rapid clumping, cessation of motion except in a few single bacilli. |
| $C^{10}$ | " | — | Slow massing of the bacilli, very little loss of motion. |
| $C^{11}$ | " | + | Slow clumping of the bacilli, with almost complete cessation of motion. |
| $D^1$ | " | — | No indications of a reaction. |
| $D^2$ | " | — | " |
| $D^3$ | " | — | " |
| $D^5$ | " | — | " |
| $D^6$ | " | — | " |
| $D^7$ | " | — | " |
| $D^8$ | " | — | " |
| $D^9$ | " | — | " |
| $D^{10}$ | " | — | " |
| $D^{11}$ | " | — | " |
| $D^{12}$ | " | — | " |
| 1 | Atypical colon bacillus obtained from well-water. | + | Characteristic clumping, with cessation of motion except in a few single bacilli, and they do not possess much translatory power. |
| 2 | Atypical colon bacillus obtained from well-water. | — | Same appearance as the control hanging-drop. |
| 3 | Atypical colon bacillus obtained from well-water. | — | Immediate clumping of a considerable portion of the bacilli. Movement of whole masses, and single bacilli are actively motile. |
| 4 | Atypical colon bacillus from well-water. | — | Slight indications of clumping. |
| 5 | Atypical colon bacillus from well-water. | — | Some clumping, but all of the bacilli remain motile. |
| 6 | Atypical colon bacillus from well-water. | — | Same appearance as the control drop. |
| 7 | Atypical colon bacillus from river-water. | — | Agglutination in masses, with considerable cessation of motility throughout the culture. |
| 8 | Atypical colon bacillus from spring-water. | — | Same appearance as the control drop. |
| 9 | Culture of the lactis aerogenes group from spring-water. | — | The bacilli gathered on the surface next the cover-slip and showed much loss of motion. Finally they dispersed and were actively motile. |
| 10 | Culture of the bacillus cloacæ from well-water. | — | Decided clumping, but active movement in both the clumps and the single bacilli. |
| 1 | River-water. | — | Indications of clumping shown by agglutination of from 15 to 20 bacilli, but active motion continues over the entire field. |
| 2 | Animal tissues. | — | Agglutination in small masses, but the bacilli are actively motile. |

TABLE IX.—*Continued.*

| Culture. | Source of culture. | Results of test with typhoid blood by the dry method of Johnston. | Remarks. |
|---|---|---|---|
| 3 | Human feces. | — | Quite large masses are formed which move from place to place; single bacilli very motile. |
| 4 | River-water. | — | Formation of quite large masses, with but little cessation of motility. |
| 5 | River-water. | — | Grouping into small masses, with but little cessation of motility. |
| 6 | River-water. | ± | Formation of small clumps, and almost entire cessation of motility. |
| 7 | From pus in abscess of abdomen. | — | Immediate formation of small clumps, cessation of motion in clumps, but single bacilli are motile. |
| 8 | Normal human feces. | — | Formation of clumps in which the bacilli are motionless. Single bacilli very actively motile. |
| 9 | From the colon at an autopsy of a case of nephritis. | + | Clumping in large masses, with cessation of motion except in an occasional bacillus. Reaction as characteristic as that of a typical typhoid culture. |

# A STUDY OF THE ACTION OF ACONITIN ON THE MAMMALIAN HEART AND CIRCULATION.

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Plates XLII and XLIII.

In the series of studies on the action of certain poisons on the mammalian heart which has been pursued during the last few years in this laboratory, that of aconitin has been my share. Circumstances induce me to publish my results now, although I must anticipate somewhat the publication of some of the physiological facts upon which their explanation is based.

The action of aconitin on the mammalian circulation has been hitherto examined only by the manometric method, from which no accurate deductions as to the real behavior of the heart are possible. The frog's heart has been observed repeatedly; but its reaction to aconitin seems to throw but little light on the complex blood-pressure tracings, and, in spite of the manifest anxiety of many of the authors to bring them into accord, there is every evidence that as great divergence exists in the behavior of the amphibian and the mammalian heart under aconitin as under the members of the digitalis series.* There are, undoubtedly, broad, general resemblances between the reaction of the two organs, but the more closely the subject is investigated the greater reserve seems necessary in drawing conclusions as to the behavior of the one from that of the other.

Böhm,† working with frogs' hearts, observed, immediately after the injection of the drug, slight quickening of the heart's rhythm, which was soon followed by slowing. In a few minutes the heart became irregular and finally stopped in diastole. The ventricle ceased to contract before the auricle, which continued to make rhythmical movements

* Cushny. *The Journal of Experimental Medicine*, ii, 233-299.
† Böhm. Studien über Herzgifte. Würzburg, 1871.

for some time afterwards. Böhm ascribed the slowing to stimulation of the inhibitory nervous mechanism of the heart. But he holds that towards the end the vagus loses all control over the heart movements, and the irregularity and final standstill are therefore due to direct action on the heart muscle.

Schroff* described similar phenomena in the mammalian heart. He observed slight quickening, soon followed by slowing, and finally a doubling of the beat, which eventually became very irregular and very weak, in consequence of which the blood pressure gradually fell to zero.

Achscharumow† observed the same phenomena as Schroff, and ascribed the slowing to stimulation of the vagus centres in the medulla oblongata. He demonstrated that after section of the vagi and the administration of atropin no slowing followed the injection of aconitin.

Wartmann,‡ in quite an extended study of the action of aconitin on the mammalian heart, observed that it was generally slightly quickened at first, but that this acceleration was soon followed by gradual slowing of the heart's rhythm, together with a corresponding fall in the blood pressure. In the blood-pressure curves the descending lines, which correspond to the diastole of the heart, often sank much below the average. Immediately after such a sinking, which corresponded to a momentary diastolic standstill of the heart, the blood-pressure curve again returned to its former level; but after several regular beats it fell again as before. These symptoms gradually increased, the sinkings of the curve becoming more frequent and deeper, and the pulse slower, often falling to one-third of its original rate. Finally the diastolic stoppages of the heart were followed by periodic quickening of its rate, during one of which the heart was liable to stop in diastole. In other experiments these periods of quickening gradually became more frequent and longer, finally supplanting the periods of slowing, when the heart soon stopped in diastole. Such action he alleged can be caused by short, often-repeated stimulations of the vagus; but, as the same phenomena appeared after section of the vagi or after the administration of atropin, or both, he ascribed them to a direct action on the heart muscle.

Giulini,§ Rosendahl,|| Leonides¶ and others have confirmed the obser-

* Schroff. Einiges über Aconitum in pharmakognostischer, toxikologischer und pharmakologischer Hinsicht. *Prager Vierteljahresschrift*, xlii, 129-184.

† *Archiv für Anatomie und Physiologie*, Jahrg. 1866.

‡ Studien über Aconit und Aconitin. Inaug. Dissert., Würzburg, 1883.

§ Inaug. Dissert. Nürnberg, 1876.

|| Arbeiten des Pharmakologischen Instituts zu Dorpat. Kobert, xi-xii, 1-118.

¶ Virchow's *Archiv*, vii, 438-478, 1854.

vations of Wartmann, but do not seem to have thrown any further light on the reaction of the heart to aconitin.

In my work I made use of the modified myocardiograph employed by Cushny in his work on the digitalis series and described in our paper on electric stimulation of the heart. In some cases the blood pressure was recorded by the ordinary mercury manometer. Dogs were used in all cases, cats and rabbits having proved unsatisfactory. The animals were anæsthetized by administering morphin (0.3 gramme) and acetone-chloroform (2 grammes). The apparatus was generally applied to the right auricle and the right ventricle, but enough tracings were taken from the left side to show that the effects were identical. Aconitin (Boehringer und Soehne and Schuchardt) was used, dissolved with the help of a few drops of hydrochloric acid, and was invariably injected into the saphenous vein.

In general terms the action of aconitin on the mammalian heart, as indicated by myocardiographic tracings, can be divided into two stages:

1. As recorded by most observers and as shown in Experiment I, during the first part of aconitin poisoning the heart's rhythm is generally slowed. The relaxations of the ventricle are little changed, perhaps a little increased, and the diastolic pauses are prolonged. The systolic contractions are weakened. In the auricle the relaxations are little affected, but the diastolic pauses are often much longer than in the ventricle. Its systolic contractions are greatly weakened, and in some experiments they are entirely suppressed, the auricle remaining in diastole. In such cases the ventricle assumes a very slow spontaneous rhythm. If the drug be administered in small doses, *e. g.* $\frac{1}{20}$ mg. every five minutes for fifteen or twenty minutes, the rhythm can be reduced to one-half, and in some cases to one-third, of its original rate, and the blood pressure lowered to a corresponding degree.

*Experiment I, Jan. 10.*—Myocardiographic tracing from dog's heart (right ventricle). Sufficient curare was given to allay all muscular movements. Vagi tested and found irritable.

| Time. | Distance of diastole from base line. | Distance of systole from base line. | Excursion of the writing lever. | Number of contractions in 10 seconds. | Height of the blood pressure from base line. | Remarks. |
|---|---|---|---|---|---|---|
| 2 h. 53 m. | 6.8 | 4.4 | 2.4 | 17 | 4.6 | |
| " 61 " | — | — | — | — | — | $\frac{1}{10}$ mg. aconitin injected. |
| " 61¼ " | 6.8 | 4.4 | 2.4 | 17 | 4.6 | |
| " 62 " | 6.8 | 4.5 | 2.3 | 16 | 4 | |
| " 64 " | 6.8 | 4.6 | 2.2 | 15 | 3.4 | |
| " 67 " | 6.8 | 4.7 | 2.1 | 14½ | 3 | |
| " 69 " | 6.8 | 5.0 | 1.8 | 14 | 3 | |
| " 71 " | — | — | — | — | — | $\frac{1}{10}$ mg. aconitin injected. |
| " 71½ " | 6.7 | 5.0 | 1.7 | 13 | 2.8 | |
| " 72 " | 6.7 | 4.9 | 1.8 | 12 | 2.8 | |
| " 73 " | 6.8 | 4.9 | 1.9 | 12 | 2.8 | |
| " 75 " | 6.9 | 5 | 1.9 | 12 | 2.8 | |
| " 78 " | 7.0 | 5 | 2.0 | 12 | 2.8 | |
| " 81 " | 7.0 | 5 | 2.0 | 10 | 2.0 | |

In this experiment the diastolic relaxations were somewhat increased, while the systolic contractions were decreased. The rate of the heart's rhythm was reduced from 17 contractions in 10 seconds to 10, and the blood pressure fell from 92 mm. of mercury to 40 mm.

2. After several small doses, that is, when about $\frac{1}{4}$ mg. has entered the circulation, the heart passes into the irregular stage described by Wartmann. As a general rule irregularity appears simultaneously in the auricle and ventricle as alternate large and small contractions (Plate XLII, Fig. 1). The contractions may not only vary in size, but the interval elapsing between them may also differ, the smaller contractions following closely on the larger and being followed by a pause of some length. In the auricle the relaxation is complete before the larger contraction and is almost complete before the smaller, while in the ventricle the relaxations between the large and small contractions may be exceedingly small. In the blood-pressure tracings, however, the smaller contractions may cause only a very slight undulation, so that the heart's rhythm may seem to be only one-half as rapid as before this type sets in. The larger contractions

cause a great wave in the manometric curve, but the average pressure at this time is considerably lower than in the previous stage. Occasionally the ventricle may seem to undergo only a somewhat prolonged contraction and to offer no renewed systole corresponding to the smaller auricular one, so that the ventricular rhythm may appear to be only half as rapid as the auricular. Examined more closely, it is seen that at the beginning of the ventricular relaxation its contraction is renewed by the extra impulse from the auricle striking it at that point, so that the arhythmia is only apparent.

During this phase of alternate large and small contractions the rhythm of the heart is practically the same as in the first stage; but somewhat later a very rapid rhythm sets in and the contractions all assume the character of the smaller contractions already described (Plate XLII, Fig. 1). In the auricle the relaxations are fairly perfect, but the contractions may not reach half-way to the line of systole of the normal heart. In the ventricle the maximum shortening may be almost equal to that of the normal heart, but the muscle does not relax fully, so that while both auricle and ventricle beat rapidly and regularly, the former departs but little from the normal position of diastole, the latter from that of systole. This may continue for some time, as was the case in the experiment from which Fig. 1 (Plate XLII) was taken. Frequently the auricle and ventricle tracings show alternately simultaneous periods of quickening and slowing. During the periods of rapid rhythm, which generally continue for from five to ten beats, the beats assume the rhythm of the smaller contractions already described, but differ from those seen in Fig. 1 in the greater amplitude of their excursions in both directions. In fact the relaxation and contraction of both auricle and ventricle may be little less complete than in their normal condition. Following this the heart gives several slow contractions, relaxing to its full extent and contracting to the line of its normal systole (Plate XLII, Fig. 2).

The duration of these alternate rhythms varies considerably, each sometimes lasting only for four or five contractions, while at other times several seconds, sometimes several minutes, of rapid contractions may be followed by a short period of slower rhythm. Not in-

frequently during these rapid periods both auricle and ventricle undergo periodic variations in the extent of systole and diastole resembling those seen in hearts under the influence of digitalis. Thus the auricle may contract generally from a position of incomplete relaxation, but every four or five beats a fuller diastole occurs.

In the second stage every contraction of the ventricle, hitherto described, has been preceded by one of the auricle, and the auriculo-ventricular rhythm has remained unaltered, the interval between the contractions of the two parts being of normal length. Often, however, another form of irregularity sets in either immediately after the first stage or during one of the periods of slower contraction already described in the second stage. Here an occasional contraction of the ventricle occurs without any corresponding auricular contraction, and another alternate series may be formed, the larger systoles of which correspond to the auricular contractions, while the smaller ones are apparently of independent origin (Plate XLII, Fig. 5). These idioventricular contractions, as a general rule, appear as short, quick systoles, originating about midway in the ventricular relaxation; but their point of origin is very variable. Later, the distinction between the alternate beats of the ventricle may be lost and the larger ones assume the character of the idioventricular contractions, and a fairly rapid rhythm almost double the rhythm of the larger systoles sets in (Plate XLII, Fig. 4). The auricle may continue beating in its former rhythm as in Fig. 5, or it may assume the rapid rhythm of the ventricle; or, again, it may cease to contract altogether, as in Fig. 6 (Plate XLII). The ventricle, after continuing for some time in its rapid rhythm, may suddenly return to a slow rhythm, with more complete contractions and relaxations (Plate XLIII, Fig. 1). More frequently, however, the rapid rhythm is interrupted at intervals by a complete relaxation and a long diastolic pause (Plate XLII, Fig. 3). Here again the condition of the auricle is variable, but in both cases it is generally weak and irregular. Again, the auricle and ventricle may assume independent rhythms, the ventricle beating slowly and fairly regularly, while the auricle takes on a more rapid rhythm and is also fairly regular (Plate XLIII, Fig. 2).

After these forms have persisted for some time a gradual dilatation of the ventricle occurs, its contractions becoming extremely irregular, both in force and in form, while the rhythm continues to accelerate. Eventually it passes into delirium cordis and remains quivering for some time in a condition of the utmost distension. The auricle may have passed into delirium previously, and this is probably the most common condition. On the other hand the ventricle may pass into delirium during a period of slow, regular auricular rhythm, and in this case the latter may persist for some time afterwards. Again, if immediately before the failure of the ventricle, both have been contracting equally rapidly and strongly, the auricle may continue to contract afterwards, but at a much slower rate. After the delirium of the ventricle the auricle can generally be restored to regular contractions by cutting into one of the great veins and allowing it to free itself of its contents.

During the second stage the blood pressure varies, corresponding to the different states of the heart. When the heart was contracting as represented in Plate XLII, Fig. 1, the blood pressure was necessarily very low, owing to the slow rhythm of the larger contractions, the smaller contractions expelling very little blood into the aorta. Here the curves were characterized by alternating great and small oscillations. Sometimes the small systoles were not visible in the pulse wave, the pulse only indicating half of the heart's contractions. The blood pressure continued low, even after the heart had assumed the rapid rhythm shown later in the same tracing, owing to the incompleteness of the contractions and the small output. But when the heart assumed a periodic rhythm, as shown in Plate XLII, Fig. 2, the pressure rose during the periods of quickening and fell during the slow periods. Again, when it was beating as represented in Plate XLII, Fig. 3, the curves were characterized by marked falls and momentary standstills corresponding to the diastolic pauses seen in the tracing. This accounts for the phenomena described by Wartmann. During the latter part of aconitin poisoning, when the ventricle was generally beating rapidly and gradually dilating, the blood pressure continued high, but when the dilatation became so great as to permit of a very small contraction it gradually fell to zero.

These two stages invariably occur in the dog's heart poisoned with aconitin. The duration of each varies with the quantity administered. The slowing in the first stage increased with the quantity of the drug injected, but when about $\frac{1}{4}$ milligramme was injected at once the stage was extremely short, the irregular rhythm setting in almost immediately. The duration of the second stage was extremely variable, delirium occurring a few minutes after the first signs of irregularity in some cases, while in others almost every form of irregularity was seen during 30 to 40 minutes. The most uniform results were got in experiments in which $\frac{1}{20}$ milligramme was injected every ten or fifteen minutes. But even with the greatest caution in the administration of the drug it is extremely difficult or impossible to anticipate what form of irregularity may result. The slowing of the cardiac rhythm during the first stage is undoubtedly due to inhibition, as earlier authors have stated, for it is entirely removed by atropin in sufficient quantities to paralyze the vagus. After section of the vagi in the neck I have observed a slight slowing, which would indicate that the peripheral inhibitory mechanism was involved in the result. This slowing of the rhythm was exceedingly small compared with that produced when the nerves were intact, so that the chief factor in the result seems to be the stimulation of the inhibitory centres in the medulla oblongata, although this is probably reinforced by peripheral action.

After the paralysis of the inhibitory mechanism by atropin the first effects produced by aconitin were irregularities corresponding to those described in the second stage. The action of aconitin in the first or therapeutic stage would, therefore, seem due entirely to its stimulating action on the inhibitory mechanism of the heart. It does not possess any action whatever on the heart muscle at first. This was confirmed by experiments in which the vagi were divided during the slowing when the contractions resumed their normal form and rate.

It may be stated here that the peripheral inhibitory mechanism is not paralyzed by aconitin during the irregular stage, as is generally stated. On stimulation of the vagus during the latest phases of irregularity I have repeatedly observed the characteristic changes in

the form of the contractions, although no diminution in the rate occurred.

The irregularities of the second stage present many points of similarity to those described by Cushny* during the second stage of digitalis action, but are on the whole more easy to interpret. I have, therefore, adopted his explanation that they are due to the interference of two rhythms in the heart muscle, and shall attempt to explain them on this basis. We have succeeded in imitating many of the forms by electric stimulation of the different divisions of the heart (compare Cushny and Matthews†). Thus in Plate XLII, Fig. 1, the movements of both auricle and ventricle correspond to those produced by an electric shock applied to the former just before the natural contraction would occur (compare Plate XLIII, Fig. 3). The smaller contractions in this case would be produced by the electric shocks, the larger ones by the natural impulse descending from the vena cava. The same effects would be produced by an electric shock reaching the ventricle soon after the spontaneous contraction, but the shock would have to be a very much stronger one. If the irritability of the auricle were much increased it may be assumed that it would also give rise to a contraction, and this contraction would be most liable to occur at the moment when the irritability reaches its highest point, that is, just before the spontaneous contraction. I think, therefore, that we are justified in considering the irregularities of the beginning of Fig. 1 (Plate XLII) as due to an increased irritability of the auricle. At first this irritability is exhausted by the effort of contraction, and the auricle, therefore, remains quiescent until the natural venous stimulus arrives, so that we have an alternation of venous and idioauricular impulses reaching the auricle and causing alternately short intervals and weak contractions, and longer intervals with stronger contractions. Later, however, as shown also in Fig. 1, the auricular irritability increases still further, so that the rhythm becomes wholly idioauricular. As I have stated, the same results may be got in some hearts from electric stimulation of the ventricle, but it seems

* Loc. cit.

† *Journal of Physiology*, xxi, No. 3.

unlikely that an idioventricular contraction should commence at a point where the muscular irritability is exceedingly low, rather than where it reaches its highest development—just before the natural contraction.

The ventricular origin of the smaller contractions of Fig. 1 would involve, therefore, a sudden increase in the irritability at the commencement of the relaxation, while the explanation I have given requires only a slight increase in the natural irritability of the auricle without any such disturbance. Each contraction of the auricle is, of course, propagated to the ventricle and sets up a contraction there. The rapid rhythm of the heart due to this idioauricular contraction occasionally disappears and a slow rhythm takes its place (Plate XLIII, Fig. 1). This would seem to be due to a return of the venous rhythm. The abnormal irritability of the auricle may be exhausted from the rapid rhythm and for a time may be insufficient to maintain a rhythm of its own, and the heart therefore takes up again the ordinary sequence in its contraction derived from the vena cava. After a time the irritability recovers enough to institute a new series of rapid contractions. During this alternating series of rapid contractions the excursions are much larger, however, than when they first commenced, and for this increase in the energy of the cardiac muscle I am unable at present to offer any explanation. Another explanation of the alternation of idioauricular and venous impulses would be a variation of the inhibitory activity, but these occur also after atropin, so that this explanation is excluded.

In another type a similar action is manifested, but here the increase in the irritability occurs in the ventricle, not in the auricle. The former, therefore, contracts doubly from its own impulses and from those received from the auricle, and the same alternations of small and large contractions occur.

Eventually the irritability becomes great enough to supply regular impulses and the ventricle beats regularly and rapidly, the excursions increasing in size as in the auricle. The effects on the auricle of the independent ventricular rhythm vary according to the conditions of the auriculo-ventricular connection. If this be impermeable for ven-

tricular impulses, the two divisions may remain beating at altogether different rates—the ventricle in its own, the auricle in the venous rhythm. If the connection be permeable, the auricle may assume the ventricular rhythm, and the heart rhythm is then completely reversed. On the other hand the auricle may suddenly pass into standstill and delirium. This occurs also during electric stimulation of the heart when, after an independent ventricular rhythm has been elicited, the auricle or venous sinus is stimulated, and seems to be due to the impulses reaching the auricle from each side. In aconitin poisoning it may be due to the auricle receiving impulses from the ventricle and also originating spontaneous contractions. As has been stated, the idioauricular contractions are, however, intermittent, and the auricular delirium therefore often alternates with periods of fairly strong contractions.

Another form is occasionally seen in which the auricular rhythm is irregular—two or three contractions of a slow rate being followed by one or more of the rapid ventricular rhythm. This would seem to be due to a partial block between the auricle and ventricle, one or two impulses passing through it and exhausting its conductivity so that the following contractions are caused by descending impulses and are therefore of slower rhythm.

Tracings, such as are represented in Plate XLII, Fig. 3, in which the rapid ventricular movements alternate with long diastolic pauses, are probably to be explained by variations in the irritability, which is at one time sufficient to cause idioventricular rhythm, while at another the ventricle has to fall back on the slow venous movements. Another probable explanation is that the ventricle, owing to its rapid rhythm, soon exhausts itself, and to compensate for this rapid expenditure of energy it gives, after every third or fourth beat, a long diastolic pause (" compensatory pause ").

The final delirium of the ventricle is also due to the ever increasing irritability of the muscle, and completes the parallel between the effects of aconitin and of electric stimulation. The ventricle may pass into this condition gradually, so that it really is impossible to draw any hard and fast lines between the idioventricular rhythm and the fibril-

lary contractions. The auricle often recovers from delirium, but I have never seen complete recovery of the ventricle from this condition, although occasionally a few rapid movements exactly similar to those occurring before delirium were often observed after it had lasted several seconds.

During the final stage the blood pressure is low and irregular. When the ventricle momentarily recovers from a condition almost resembling delirium it rises, only to fall again when the fibrillary contractions return. This rising and falling may continue for some seconds, but when real delirium sets in the pressure falls to zero.

The form of irregularity produced by aconitin in the second stage seems to depend upon whether it increases the irritability of the auricle or that of the ventricle to the greater extent, and upon the resistance offered to the passage of impulses between the auricle and the ventricle. It is possible that when the inhibitory apparatus is intact both of these factors may be influenced by the stimulation of the vagus centre in the medulla, but even after atropin the same varieties may be observed in the irregularities elicited. That the resistance to the passage of impulses may vary in different hearts is generally recognized, and we have observed repeatedly a complete block in the auriculo-ventricular connection as far as the passage backward of ventricular impulses was concerned.

## CONCLUSIONS.

The action of aconitin on the dog's heart, therefore, seems to consist in:

1. A stimulation of the inhibitory mechanism, especially of the centres in the medulla oblongata.

2. An increase in the irritability of the muscle of the auricle and the ventricle, which leads to independent contractions of one or both of these divisions and culminates in fibrillary contractions in the ventricle.

The first of these is the only effect seen in the therapeutic use of the drug, and aconitin may, therefore, be considered to be indicated when it is desirable to stimulate the inhibitory centre without acting on the heart muscle. Of course it has a further effect on the circula-

tion through the stimulation of the vaso-motor centre, but this would appear to be of minor importance.

## DESCRIPTION OF PLATES XLII AND XLIII.

All the tracings in the accompanying plates read from left to right. In all figures representing simultaneous tracings from the right auricle and the right ventricle, the upper one belongs to the ventricle, the lower one to the auricle. The lever descends during systole and ascends during diastole in all cases.

### PLATE XLII.

Fig. 1 represents simultaneous tracings from the right auricle and the right ventricle, and shows one of the first forms of irregularity produced by the action of aconitin on the heart muscle.

Fig. 2 represents a tracing from the right ventricle and shows the alternating slow and rapid rhythm.

Fig. 3 represents a tracing from the right ventricle, and shows a rapid rhythm alternating after every third or fourth beat with a very complete relaxation and a prolonged diastolic pause.

Fig. 4 represents a tracing from the right ventricle, and shows a sudden change from alternating idioventricular systoles to idioventricular contractions.

Figs. 5 and 6 represent simultaneous tracings from the right auricle and the right ventricle, and show idioventricular systoles alternating with venous systoles. Contrast the condition of the auricle in these two.

### PLATE XLIII.

Fig. 1 represents a sudden change from a rapid idioventricular rhythm to a slow seemingly venous rhythm.

Fig. 2 represents simultaneous tracings from the right auricle and the right ventricle, and shows the auricle and the ventricle beating in independent rhythms.

Fig. 3 represents simultaneous tracings from the right auricle and the right ventricle of a heart in which the auricle was stimulated by an electric shock (" break ") at the point in each of its relaxations marked by the small cross lines seen in the tracing. This tracing shows the effect of electric stimulation of the auricle, not only on the auricle itself but also on the ventricle, and is intended to show the similarity in the contractions of an auricle stimulated by electric shocks, and one having its irritability increased by aconitin, as in Plate XLII, Fig. 1.

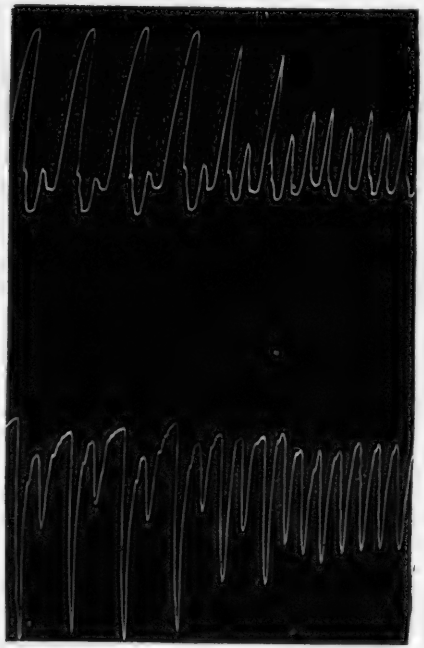

Fig. 1.

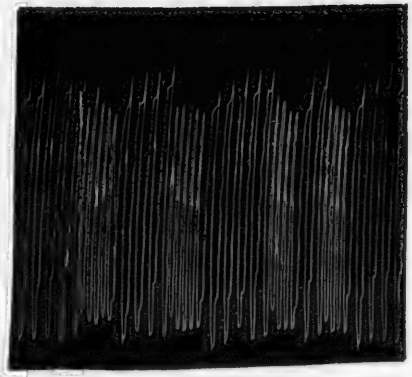

Fig. 2.

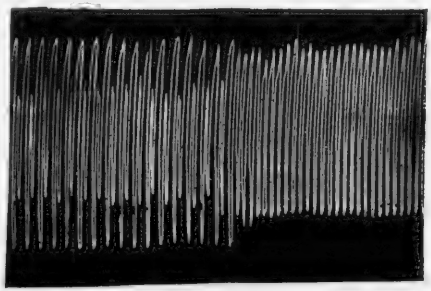

Fig. 4.

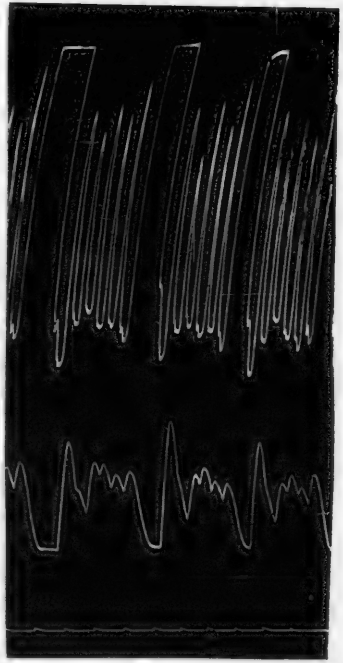

Fig. 3.

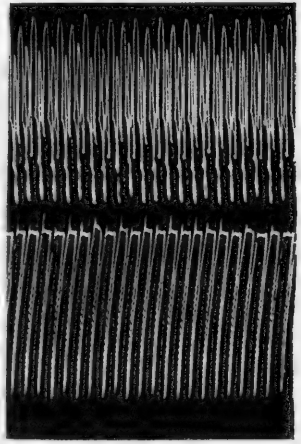

Fig. 5.

Fig. 6.

Fig. 1.

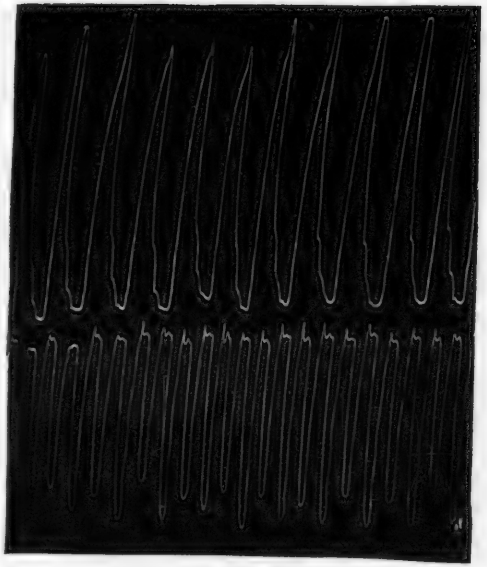

Fig. 2.

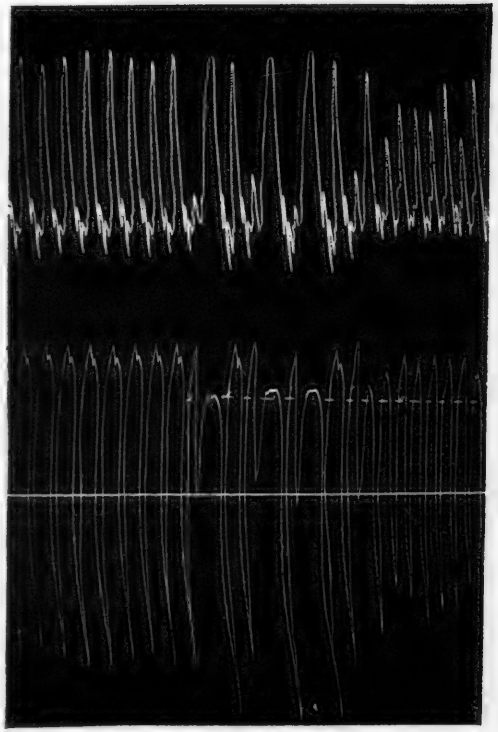

Fig. 3.